Increased Survival, Proliferation, and Migration in Metastatic Human Pancreatic Tumor Cells Expressing Functional CXCR4

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ABSTRACT

In this study, we have evaluated 11 pancreatic tumor cell lines and tumor cells from surgical samples of patients with pancreatic adenocarcinoma for expression of the chemokine receptor CXCR4. Six of 11 cell lines expressed detectable mRNA of CXCR4, with three cell lines (AsPC1, Capan1, and Hs766T) having substantial amounts of transcripts. Expression was higher in lines derived from metastatic lesions compared with those derived from primary tumors. Different inflammatory cytokines did not modify expression, whereas IFN-γ down-regulated and hypoxia up-regulated CXCR4 transcripts. Transcript expression was associated with surface expression in pancreatic carcinoma cell lines. All surgical carcinoma samples tested expressed higher levels of CXCR4 than normal pancreatic ducts, which were used as reference tissue. The chemokine CXCL12 induced chemotaxis in CXCR4-positive pancreatic carcinoma cell lines, which was inhibited by anti-CXCR4 monoclonal antibody and by the antagonist AMD3100. Transendothelial migration, Matrigel invasion, and activation of matrix metalloproteases were also enhanced by CXCL12. In CXCR4-positive cell lines, CXCL12 stimulated cell proliferation. The cell line Hs766T produces high levels of CXCL12, and addition of exogenous CXCL12 induced chemotaxis in CXCR4-positive pancreatic carcinoma cell lines. All surgical carcinoma samples tested expressed higher levels of CXCR4 than normal pancreatic ducts, which were used as reference tissue. The chemokine CXCL12 induced chemotaxis in CXCR4-positive pancreatic carcinoma cell lines, which was inhibited by anti-CXCR4 monoclonal antibody and by the antagonist AMD3100. Transendothelial migration, Matrigel invasion, and activation of matrix metalloproteases were also enhanced by CXCL12. In CXCR4-positive cell lines, CXCL12 stimulated cell proliferation. The cell line Hs766T produces high levels of CXCL12, and addition of exogenous CXCL12 inhibited apoptosis induced by serum starvation. These results indicate that the CXCR4 receptor is frequently expressed in metastatic pancreatic tumor cells. CXCR4 not only stimulates cell motility and invasion but also promotes survival and proliferation. Strategies to target CXCR4 expressed on tumor cells may be of benefit in patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a highly invasive disease: >80% of the patients show tumors locally extended beyond the pancreas and metastases in regional lymph nodes. Its aggressive behavior and the lack of sensitivity to most treatment options render this tumor a major cause of cancer-related death, with a mortality rate virtually equal to its incidence: 10 patients over 100,000 individuals in Western countries. From these data, it appears clear that a better understanding of the fundamental nature of pancreatic cancer is needed to improve the clinical outcome (1, 2).

In the last two decades, there has been increasing evidence for a role of chemokines in tumor biology (3–8). Chemokines were first described as small peptides controlling cell migration, especially that of leukocytes during inflammation and immune response. Thereafter, a broad spectrum of biological activities has been described as chemokine-regulated, which also affects tumors and their microenvironment. Indeed, tumors constitutively produce chemokines, and there is strong evidence that tumor-derived chemokines are major determinants of the leukocyte infiltrate of neoplasms (7, 9–13). We recently reported that pancreatic cancer produces CCL2, whose levels correlate with tumor macrophage infiltration (14). Some chemokines may enhance innate and specific host immunity against tumors, but on the other hand, other chemokines may contribute to escape from the immune system by recruiting Th2 effectors and regulatory T cells (15). Chemokines are potent activators of macrophage-mediated digestion of extracellular matrix and therefore may indirectly favor tumor dissemination. Finally, chemokines may also favor tumor growth by directly promoting cell proliferation, migration, or neovascularization in tumor tissue. For instance, CXCL8 and CXCL1 have been implicated as endogenous growth-stimulating factors in malignant melanoma, as well as in tumors of different histology (3–7, 13, 16, 17).

Chemokines act through specific 7-transmembrane receptors coupled to G proteins (18). Expression of chemokine receptors on the surface of tumor cells of epithelial origin has been studied to a much lesser extent compared with ligand production. Earlier studies pointed out that some tumor cell lines migrated in response to chemokines and that antibodies against CXCR2 were able to inhibit the growth of melanoma cells in vitro (19, 20). Recently, it was showed that chemokine receptors expressed by tumor cells induce cell migration and associated to distant metastasis in vivo, suggesting that chemokines may control tumor dissemination. Breast cancer cells and melanoma express CXCR4 and CCR7, which bind to CXCL12 and CCL19/CCL21, respectively (21). Melanoma cells also express CCR10 (receptor for CCL27; ref. 21) and, as shown several years ago, also CXCR1 and CXCR2 (receptors for CXCL8; refs. 19, 22, 23). Gastric tumors were reported to express CCR7 (24), and tumors derived from the ovaries, kidney, prostate and small-cell lung cancer, mainly express CXCR4 (25–28). In pancreatic cancer, expression of CCR6 and its ligand chemokine (CCL20) has been reported previously (29). Recently, CXCR4 expression has been observed both in pancreatic cell lines and in primary tumors (30, 31). However, functional activity of CXCR4 was tested only in one cell line in chemotaxis assays.

In this study, we have analyzed the expression of CXCR4 in a large panel of 11 pancreatic tumor cell lines and in freshly isolated surgical samples. CXCR4 was expressed most prominently in cell lines derived from metastatic tumors. On CXCR4-positive cell lines, CXCL12 not only enhanced chemotaxis, transendothelial migration, and Matrigel invasion but also stimulated tumor cell proliferation and was able to protect from serum deprivation-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Tissues. Human pancreatic carcinoma cell lines AsPC-1, Capan1, MiaPaCa-2, Panc-1, and Hs766T were purchased from the American Type Culture Collection (Manassas, VA), and A8184, PT45, HPAF, CFPAC, PaCa44, were kindly provided by Professor Aldo Scarpa (Department of
Pathology, University of Verona, Verona, Italy). Four cell lines were obtained from primary tumors (PT45, PaCa44, MiaPaCa2, and Panc1), three from ascites (AsPC 1, A8184, and HPAF), and four from metastasis (T3M4, CFPAC, Capan1, and HS766T). The immortalized epithelial cell line derived from normal human pancreatic ducts HPD6E6, kindly obtained from Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada), has been previously shown to maintain the phenotypic and genotypic characteristics of normal human pancreatic ducts (32). The cell lines were maintained in DMEM (Life Technologies, Inc., Paisley, Scotland, United Kingdom) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and in a humid atmosphere of 5% CO2/95% air. Where indicated, cell lines were treated with interleukin (IL)-1β (10 ng/mL), tumor necrosis factor α (10 ng/mL), IFN-γ (500 units/mL) (all from Peprotech, Rocky Hill, NJ) for 8 hours. Hoxpia was generated by culturing cells for 8 hours in an atmosphere-controlled culture chamber (Bello Glass) containing a gas mixture composed of 94% N2, 5% CO2 and 1% O2. Tumor cells were also treated with desferoxamine (0.4 mM/L), an iron chelator recognized as an hypoxia-mimicking compound (33).

Primary tumors from surgical specimens were enzymatically digested with collagenase-hyaluronidase for 2 hours at 37°C as described previously (14). Cells were plated on Primaria plates (Falcon, Franklin Lakes, NJ) at a density of 1 × 105 cells/mL and maintained in DMEM supplemented with 10% fetal bovine serum. Tumor cells grew as adherent cells with epithelial morphology and were ≈95% positive for expression of cytokeratins 7 and 18. After 24 to 48 hours, cells were prepared for RNA extraction and performed with Trizol (Life Technologies, Inc.), following the manufacturer’s instructions. Freshly isolated human epithelial pancreatic duct cells were obtained from the pancreas of a multiorgan donor in irreversible coma by enzymatic digestion, as described above for primary tumors.

Reverse Transcription-PCR and Real-Time PCR. Total RNA extraction from fresh or cultured cells was done with Trizol.

cDNA was synthesized by random priming from 1 μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. The following primers were used for the subsequent PCR: human CXC4r4 (sense, 5'-AGCTGTGGCTGAAAGGTT-GGTCTATG-3'; antisense, 5'-GCCCTTCTGGGCGCCTTGGATTGTG-3'); human β-actin (sense, 5'-AAGTAGCAGCAGATGTGTTTG-3'; antisense, 5'-GGAGGCTTTGCTGTTCCT-3'). PCR was done with AmpliTaq DNA Polymerase (Applied Biosystems) following the manufacturer’s instructions. Cycling conditions: 2 cycles (20 cycles for β-actin) of 1 minute at 94°C, 1 minute at 56°C, and 1 minute at 72°C. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Real-time PCR was done with SYBR Green dye and GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City CA). The sequences of primer pairs specific for each gene (Invitrogen, Milan, Italy) were designed with Primer Express Software (Applied Biosystems). Two μL of cDNA were used as the template; 12.5 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems) were mixed with template and primers. The total reaction volume was 25 μL. Cycling conditions were 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Experiments were done in triplicate for each sample.

For the CXC4r4 gene, mRNA was normalized to β-actin mRNA by subtracting the cycle threshold (Ct) value of β-actin mRNA from the Ct value of the gene (ΔCt). Fold difference (2−ΔΔCt) was calculated by comparing the ΔCt with either the ΔCt of the HPDE cell line or with that of unstimulated cells.

The sequences of primer pairs were as follows: human CXC4r (sense, 5'-CAAGGCCCCTCAGACGACCAT-3'; antisense, 5'-CCCAATGTAGTAAAG-GAGCGCCA-3'); human β-actin (sense, 5'-TCACCCACATGCGCCATCATCAGCA-3'; antisense, 5'-GAGGGAAGCGCTTCTGGCAGCTT-3').

Flow Cytometry. Pancreatic cancer cells were removed from cultured non-enzymatically, washed, resuspended in ice-cold washing buffer (PBS containing 1% human serum) and incubated with 10 μg/mL fluorescein-labeled mouse antihuman CXC4r antibody (12G5; R&D Systems, Minneapolis, MN) for 30 minutes at 4°C. Cells were analyzed with a FACScanCalibur flow cytometer.

Chemotaxis Assay. Cell migration was evaluated with a 48-well modified Boyden chamber as previously described, with minor modifications (34). The polycarbonate filter (12-μm pores size; CN10416, Neuroprobe, Baltimore, MD) was precoated with fibronectin (5 μg/mL; Sigma, St. Louis, MO); cells were resuspended at 5 × 105/mL in the appropriate medium supplemented with fetal bovine serum 1% and seeded in the upper chamber. Recombinant CXCL12 and CCL2 (Peprotech, Rocky Hill, NJ) were used as chemotactants in the lower compartment. The chambers were incubated overnight at 37°C. Results are expressed as the mean number of net migrated cells per control cells, counted in 10 microscope high power fields (magnification, ×1000). Each experiment was done in triplicate. Where indicated, cells were incubated with a blocking anti-CXCR4 monoclonal antibody (12G5, 10 μg/mL; R&D Systems) or with AMD3100 (1 μg/mL; Sigma).

Preparation of Endothelial Cells and Transmigration Assay. Human endothelial cells were obtained from umbilical vein and cultured as described previously (35). Cells were maintained in 199 medium with 20% bovine serum, supplemented with endothelial cell growth supplement (100 μg/mL; Collaborative Research, Inc., Lexington, MA) and heparin (100 μg/mL; Sigma Chemical Co., St. Louis, MO). The purity of endothelial cell cultures was checked by expression of von Willebrand factor and found to be >99% positive.

The transmigration assay was done as described previously (35). In brief, endothelial cells were grown to confluency on polyvinylpyrrolidone-free polycarbonate filters (12-μm pore) and mounted on Boyden chambers over a second filter. Cr-labeled tumoral cells were seeded in the upper compartment and co-incubated with endothelial cells monolayers for 16 hours at 37°C. Nonadherent cells were gently washed away, and adherent cells were scraped with a cotton swab. The radioactivity in the double filter and in the lower compartment referred to transmigrated cells. The adherent cells were considered to comprise both cells bound to endothelial cells, as well as those that had transmigrated.

Gelatinase Activity Assay. Pancreatic cancer cell lines were grown in serum-free X-Vivo medium and stimulated 24 hours with 300 ng/mL CXCL12. Supervantants were analyzed using a MMP Gelatinase Activity kit (Chemicon International, Inc., Temecula, CA), according to manufacturer’s instructions.

Invasion Assay. Cell invasion was examined with a 48-well modified Boyden chamber and a reconstituted extracellular matrix membrane (Matrigel; Becton Dickinson, Bedford, MA). Cell invasion chambers were prepared by carefully placing onto a polycarbonate polyvinylpyrrolidone-free filter 10 μL of Matrigel (0.5 μg/mL) and incubating at 37°C for 30 minutes to allow Matrigel polymerization, then 45 μL of cell suspension were added to each well and incubated at 37°C overnight. Results were evaluated as for the chemotaxis experiment.

Proliferation Assay. Proliferation was measured in vitro with the vital dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester, mixed isomer (CFSE; Molecular Probes, Inc., Eugene, OR). Briefly, after 12 hours of synchronization in medium without serum, cells were labeled with CFSE (5 μM/L), seeded in 12-well plates, and stimulated. After 3 days, cell division was indicated by decreased CFSE fluorescence intensity, as assessed by flow cytometry.

Apoptosis Assay and Cell Cycle Analysis. For cell cycle analysis, cells were seeded and stimulated after 12 hours to let them adhere. After 72 hours, they were collected, washed once with PBS, and fixed with 70% ethanol at −20°C for 24 hours. Fixed cells were three washes three times and incubated for 1 hour with a propidium iodide (PI) solution (20 μg/mL; Sigma Chemical Co.) containing RNaseA (0.1 μg/mL; Sigma Chemical Co.). Cells were then subjected to cell cycle analysis for determining DNA contents by flow cytometry. Cell debris were excluded on the basis of forward versus side scatter. Doubles and clumps were excluded by gating on a bivariate distribution of AUX (PI peak pulse) versus the PI integrated signal. Data from 10,000 events were collected in the final gated histograms. The cell histogram was divided into three regions according to cell cycle phase: G0/G1, G2-M, and sub-G1 peak (PI fluorescence from fractional DNA content of apoptotic cells), which defined the proportion of apoptotic cells.

The percentage of cells undergoing apoptosis was determined with fluorescein-conjugated human annexin-V (PhaMingen; BD Biosciences, San Diego, CA). Cells were plated in 12-well plates and cultured for 18 hours. Then they were incubated for 24 hours under apoptosis-inducing conditions (serum depriva- tion) with or without CXCL12 (100 ng/mL). Cells were collected and stained at room temperature in the dark for 15 minutes in 200 μL of buffer containing FITC–annexin-V (5 μL). Annexin-V+ cells correspond to apoptotic cells.

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RESULTS

**CXCR4 Expression in Pancreatic Tumor Cell Lines.** We first evaluated the expression of CXCR4 by reverse transcription-PCR in 11 established pancreatic tumor cell lines. CXCR4 mRNA expression was clearly detected in 6 of 11 lines, with different amounts of CXCR4 transcripts; in particular, Hs766T showed a very high expression, quite comparable with the expression in normal peripheral blood mononuclear cells, used as positive control (Fig. 1A). Expression of CXCR4 mRNA was tested also in an immortalized epithelial cell line derived from normal human pancreatic ducts (HPDE6). This cell line has been previously shown to maintain the phenotypic and genotypic characteristics of normal human pancreatic ducts (32). HPDE6 showed no detectable expression of CXCR4 transcripts (Fig. 1A).

To better appreciate the differences between cell lines, CXCR4 mRNA expression was evaluated by semiquantitative real-time PCR. The cell line HPDE6 was used as reference. This second analysis confirmed the expression of CXCR4 in the six positive tumor cell lines (at least 20-fold compared with HPDE6 cells). The cell lines Hs766T, AsPC1, and Capan1 showed the highest expression (1165-, 122-, and 86-fold, respectively; Fig. 1B). Interestingly, CXCR4 was more frequently expressed in cell lines originating from metastatic or ascitic lesions, compared with cell lines derived from primary tumors.

Cell-surface expression of CXCR4 was evaluated by flow cytometry. Representative phenotype profiles of the cell lines Hs766T, AsPC1, Capan1, A8184, and CFPAC are shown in Fig. 1C.

**Regulation of CXCR4 Expression in Pancreatic Tumor Cell Lines.** Cytokines are potent modulators of chemokine receptor expression and are frequently present in the tumor microenvironment. We have analyzed whether cytokines modulate the expression of CXCR4 in pancreatic tumor cells. In two cell lines tested (Hs766T and AsPC1), IL-10, IL-6, HGF, IL-1β, and tumor necrosis factor α did not modify CXCR4 expression, whereas IFN-γ consistently down-regulated CXCR4 transcripts (Fig. 1D, left panel, and data not shown). Hypoxia is a low-oxygen tension condition frequently present in tumoral necrotic areas. Our group has recently showed that hypoxia up-regulates the expression of CXCR4 in human monocytes and in ovarian cancer cells (36). Therefore, we tested pancreatic tumor cells cultured in hypoxic conditions. Tumor cell lines expressing, or non-expressing CXCR4, were tested either after culture in a low-oxygen chamber or after treatment with desferrioxamine, an hypoxia-
CXCL12 stimulates pancreatic cancer cell chemotaxis and transendothelial migration. To verify that CXCR4 is functional in pancreatic cancer, selected cell lines were tested in different functional assays. The cell line Capan1 was excluded for its characteristic to disaggregate in large clusters. Fig. 3A shows that the CXCR4-positive cell lines Hs766T, AsPC1, A8184, and CFPAC did migrate in response to CXCL12 in a classical chemotaxis assay, with an optimal response at 300 ng/mL. In contrast, the CXCR4-negative cell lines PT45 and MiaPaCa2 did not migrate to CXCL12 (Fig. 3A). We recently reported that none of these pancreatic tumor cell lines express CCR2 (14). In line with this finding, different concentrations of CCL2 did not induce chemotactic response in Hs766T or AsPC1 cells (Fig. 3A). Treatment of tumor cells with a blocking anti-CXCR4 monoclonal antibody or with the CXCR4 antagonist AMD3100 drastically inhibited cell migration in response to CXCL12 (Fig. 3B).

Tumor cell adhesion to endothelial cells and transendothelial migration are key steps in the process of tumor invasion and metastatization. CXCL12 significantly enhanced adhesion to human umbilical vascular endothelial cells and transendothelial migration of the CXCR4-positive cell line Hs766T (Fig. 3C).

CXCL12 stimulates gelatinase activity. Chemokines are potent activators of matrix metalloproteases (MMPs). Hence, it was of interest to measure the activity of MMP-2 and MMP-9 in the supernatants of CXCL12-treated tumor cells. Freshly isolated human mono-

CXCL12 stimulates pancreatic cancer cell chemotaxis and transendothelial migration. A. CXCR4-positive cell lines (Hs766T, AsPC1, CFPAC, and A8184) migrated in classical chemotaxis assays to different concentrations of CXCL12 in a dose-response manner, whereas CCL2 did not induce a chemotactic response in the cell lines Hs766T and AsPC1. The CXCR4-negative cell lines, PT45 and MiaPaCa2, did not migrate to CXCL12. Shown are net numbers of migrated cells over basal migration (in the absence of chemokine). Basal migration was 120 cells/10 high power field (HPF) for Hs766T, 23 cells/10 HPF for AsPC1, 22 cells/10 HPF for CFPAC, 25 cells/10HPF for A8184, 47 cells/10 HPF for MiaPaCa2, and 27 cells/10 HPF for PT45 (NS versus control; **P < 0.02 versus control). Values are the mean ± SE of eight replicates. One representative experiment of three performed is shown. B. Pretreatment of Hs766T cells with a blocking anti-CXCR4 monoclonal antibody (10 μg/mL) or with AMD3100 (1 μg/mL) significantly reduced cell migration in response to 300 ng/mL CXCL12 (**P < 0.01 versus control). C. CXCL12 significantly enhanced adhesion and transmigration through endothelial cells (+P < 0.02 versus control). Human umbilical vascular endothelial cells were grown on polycarbonate filters. Transendothelial migration was assessed after 16 hours of incubation in the presence of CXCL12 (300 ng/mL) in the lower compartment.

Fig. 3. CXCL12 stimulates pancreatic cancer cell chemotaxis and transendothelial migration. A. CXCR4-positive cell lines (Hs766T, AsPC1, CFPAC, and A8184) migrated in classical chemotaxis assays to different concentrations of CXCL12 in a dose-response manner, whereas CCL2 did not induce a chemotactic response in the cell lines Hs766T and AsPC1. The CXCR4-negative cell lines, PT45 and MiaPaCa2, did not migrate to CXCL12. Shown are net numbers of migrated cells over basal migration (in the absence of chemokine). Basal migration was 120 cells/10 high power field (HPF) for Hs766T, 23 cells/10 HPF for AsPC1, 22 cells/10 HPF for CFPAC, 25 cells/10HPF for A8184, 47 cells/10 HPF for MiaPaCa2, and 27 cells/10 HPF for PT45 (NS versus control; **P < 0.02 versus control). Values are the mean ± SE of eight replicates. One representative experiment of three performed is shown. B. Pretreatment of Hs766T cells with a blocking anti-CXCR4 monoclonal antibody (10 μg/mL) or with AMD3100 (1 μg/mL) significantly reduced cell migration in response to 300 ng/mL CXCL12 (**P < 0.01 versus control). C. CXCL12 significantly enhanced adhesion and transmigration through endothelial cells (+P < 0.02 versus control). Human umbilical vascular endothelial cells were grown on polycarbonate filters. Transendothelial migration was assessed after 16 hours of incubation in the presence of CXCL12 (300 ng/mL) in the lower compartment.
cytes were used as positive control. The gelatinase activity of MMPs from untreated tumor cells was very low (Fig. 4A). CXCL12 significantly increased MMP activity in Hs766T and AsPC1, with levels of activity similar or higher than human monocytes, whereas the chemokine did not affect the MMP activity in the CXCR4-negative cell line MiaPaCa2. The activity of metalloproteases is of major importance in the digestion of the extracellular matrix and has been implicated in the metastasizing ability of tumor cells. In line with the finding that CXCL12 induces MMP activity, tumor cells stimulated with CXCL12 showed enhanced ability to invade Matrigel-coated filters (Fig. 4B).

CXCL12 Stimulates Pancreatic Cancer Cell Line Proliferation. Tumors frequently produce chemokines. We first evaluated whether pancreatic cancer cells produce CXCL12. Of 11 cell lines tested, only Hs766T produced significant amounts of the chemokine (1326 pg/mL/10^6 cells).

The effect of CXCL12 on tumor cell proliferation was assessed on CFSE-labeled cells. Under optimal culture conditions (in the presence of 10% serum), addition of CXCL12 increased proliferation in the cell line AsPC1 but not in Hs766T cells, which produce the chemokine (Fig. 5A). Treatment with AMD3100 greatly inhibited spontaneous proliferation only in Hs766T cells, which is in line with the presence of the endogenous chemokine.

Under suboptimal culture conditions (absence of serum), the presence of CXCL12 greatly enhanced cell proliferation in both Hs766T and AsPC1 cell lines (Fig. 5B). This is in agreement with our finding that CXCL12 release was reduced by >90% in serum-free conditions (data not shown). The enhancing effect of CXCL12 on cell proliferation was strongly inhibited by treatment with AMD3100 (Fig. 5, A and B).

These results indicate that CXCL12 stimulates pancreatic cancer cell line proliferation, and, in at least one representative CXCR4-positive cell line, the chemokine acts as an autocrine growth factor.

CXCL12 Promotes Survival in Pancreatic Cancer Cells. An important feature of metastatic cells is the ability to regulate their survival. We therefore tested whether CXCL12 could rescue Hs766T and AsPC1 tumor cells from serum deprivation-induced death. Treatment of cells cultured in serum-free medium with CXCL12 reduced the percentage of PI-positive cells by 46% in Hs766T and by 50% in AsPC1 (n = 4, data not shown). We next performed cell cycle analysis. As shown in Fig. 6A, serum deprivation enhanced the proportion of apoptotic cells, as indicated by the sub-G1 peak detection. We also used IL-1β as an apoptotic stimulus because we previously observed that this cytokine induces apoptosis in these cell lines (14). Addition of CXCL12 (100 ng/mL) significantly reduced spontaneous DNA degradation, as shown by decrease of sub-G1 peak (from 27 to 16%) and from 11 to 4% for Hs766T and AsPC1, respectively; where IL-1β was used, from 21 to 16% and from 6 to 2%, in Hs766T and in AsPC1, respectively).

To additionally address the question whether CXCL12 regulates survival of pancreatic cancer cell lines, we evaluated the percentage of...
annexin-V+ cells under serum-deprived culture conditions. In both Hs766T and AsPC1 cell lines, treatment with CXCL12 decreased the percentage of apoptotic annexin-V+ cells (from 19 to 15% in Hs766T and from 17 to 12% in AsPC1), whereas it had no significant effect in the CXCR4-negative cell line MiaPaCa2 (Fig. 6B).

**DISCUSSION**

Despite advances in surgical, as well as medical treatments, pancreatic cancer remains a tumor with very poor prognosis. Quite uniquely, most patients die within few months of diagnosis, even in the presence of small tumor loads, with severe cachexia and anorexia and extreme pain. In addition, intrinsic properties of neoplastic cells contribute to the rapid progression and invasiveness of this tumor. Pancreatic cancer is characterized by the aberrant expression of several growth factors, enhanced angiogenesis, a marked desmoplastic reaction and resistance to apoptosis. These hallmarks influence the aggressiveness of this tumor (1, 2).

A complex network of chemokines and receptors exists in the tumor microenvironment. Chemokines are crucial determinant of the leukocyte infiltrate of tumors and exert a variety of biological functions, including regulation of angiogenesis, activation of matrix metalloproteases, growth promoting effects, and inhibition of apoptosis (3–8). In addition, functional chemokine receptors, possibly playing a role in the process of metastatization, have been described in tumors from breast, melanoma, ovaries, stomach, and other carcinomas (21, 24–27). Therefore, the expression of chemokines and receptors is of potential advantage for tumor cells and may endow them with enhanced ability to proliferate and disseminate. Pancreatic tumors have been reported to express CCR6 and its ligand CCL20 (29). More recently, Koshiba et al. (30) investigated CXCR4 in pancreatic tumors and found that mRNA was expressed in all of the five cell lines tested and that the chemokine CXCL12 induced chemotactic migration in one cell line (30, 31).

In this study, we report that 6 of 11 pancreatic tumor cell lines express considerable levels of CXCR4 transcripts, which was confirmed at the protein level in selected cell lines. We also found that surgical specimens from pancreatic adenocarcinomas express CXCR4 in higher amounts compared with an immortalized cell line derived from human pancreatic ducts (HPDE6), as well as compared with freshly isolated pancreatic ducts. The finding that tumor tissues have higher expression of CXCR4, compared with the normal counterpart, is in agreement with previous reports (25, 26, 30). We also observed a differential expression of CXCR4 in the pancreatic cancer cell lines depending on their origin. Only one of three cell lines derived from primary tumors expresses the transcript, in very low amounts, although all but one cell line derived either from ascites or metastasis express higher amounts of CXCR4 mRNA. This result suggests an association between the expression of the receptor and the metastatic potential of tumor cells.

Surprisingly, the preparation of freshly isolated pancreatic ducts that was tested had relatively high levels of CXCR4. One possible explanation is that these pancreatic ducts were isolated from a multi-
organ donor in irreversible coma. Although it is unknown whether this specific pathological condition affects the expression of chemokine receptors, it is well known, and we also confirm it in this report that hypoxia up-regulates CXCR4. It may be possible that the high CXCR4 expression is the result of hypoxic conditions surely occurred before pancreas explant. High expression of CXCR4 in pancreatic ducts has also been recently reported by Kajali et al. (37) in NOD-IFN-γ–transgenic mice, as well as in parental NOD mice. These authors also highlight the importance of the CXCL12-CXCR4 ligand-receptor axis in the survival, proliferation, and migration of ductal pancreatic cells.

Modulation of chemokine receptors has not been extensively investigated on tumor cells. It has been recently reported that hypoxia (low-oxygen tension) up-regulates CXCR4 expression in endothelial and tumor cells (36). Indeed CXCR4 is an hypoxia inducible gene, being a target of hypoxia-inducible factor 1 α (38). In line with this finding, we observed that receptor expression was enhanced in CXCR4-positive cell lines cultured under hypoxic conditions. As low-oxygen tension is likely to occur in the neoplastic mass, hypoxia-induced up-regulation of CXCR4 may have direct in vivo relevance.

To assess whether cytokines present in the tumor microenvironment could modulate CXCR4, we tested IL-10, IL-6, and hepatic growth factor (known to be produced by human pancreatic tumor and stromal cells; refs. 39, 40). CXCR4 mRNA transcripts were never affected by the above cytokines (data not shown). The inflammatory cytokines, tumor necrosis factor α and IL-1β, were also ineffective; in contrast, treatment with IFN-γ consistently reduced CXCR4 expression. It is well established that IFN-γ plays a crucial role in immune resistance against tumors (41). Therefore, the IFN-γ-mediated down-modulation of CXCR4 may contribute to inhibition of tumor growth and metastasis.

In line with other results that CXCR4 is implicated in promoting the migratory phenotype of a variety of tumors (21, 25–27, 30), we found that CXCL12 induced chemotactic response in CXCR4-positive cell lines. CXCL12-induced chemotaxis was inhibited when CXCR4 receptors were blocked by a specific monoclonal antibody or by the CXCR4 antagonist AMD3100. Tumor cell adhesion to endothelium and transendothelial migration were also enhanced by CXCL12 in CXCR4-positive pancreatic tumor cells.

Cancer dissemination can be viewed as a tissue remodeling process that involves proteolytic degradation of extracellular matrix in the surrounding normal tissue. Metalloproteases are a family of enzymes activated by chemokines, involved in the degradation of extracellular matrix and known to mediate cancer invasion and metastases (13, 42–44). Among the 11 types of metalloproteases, MMP-2 and MMP-9 participate in the degradation of type IV collagen, which is one of the major components of vascular basement membranes, whose elimination is essential to invade stroma and vessels; indeed, expression of MMP-2 and MMP-9 has been associated with venous invasion and hematogenous metastasis (45). Pancreatic cancer is strongly invasive, usually directed to large vessels, such as the portal vein, and the development of liver metastasis is generally dependent on venous invasion by primary tumor cells. In the absence of chemokines, the gelatinase activity of MMPs from untreated tumor cells was very low. In contrast, in CXCL12-treated tumor cell lines, MMPs were significantly more active. In line with these findings, CXCL12 triggered tumor cell invasion through a Matrigel layer. Collectively, these results indicate that CXCR4 expression confers tumor cells with increased motility and invasion ability.

Chemokines may have direct or indirect growth-stimulating effects on tumor cells. There is evidence that CXCL8 and CXCL1 are implicated as endogenous growth-stimulating factors in melanoma, as well as in tumors of different histologies. Our group has extensively analyzed the expression and production of different chemokines in pancreatic cancer; some of the cell lines produce CCL2, CCL5, and CXCL8, with a heterogeneous pattern (14, 46). In this work, we show that CXCL12 is produced only in HS766T, the cell line expressing highest levels of CXCR4. Interestingly, the CXCR4 antagonist AMD3100 inhibited proliferation in HS766T cells, suggesting that the endogenously produced CXCL12 may function as an autocrine or paracrine growth factor. In vivo CXCL12 can be produced by several cell types, including stromal and endothelial cells (27); moreover, this chemokine is produced in lymph nodes, where secondary localization of tumors, including pancreatic carcinoma, occurs.

In vitro experiments, CXCL12 enhanced the proliferation of the CXCR4-bearing cell lines, especially under suboptimal culture conditions. When HS766T cell line was cultured in serum-free medium, CXCL12 release was dramatically reduced and exogenous CXCL12 significantly stimulated cell growth, both in HS766T and in AsPC1. Our results of CXCL12 enhancing tumor cell proliferation is in agreement with other reports. CXCL12 stimulated the proliferation of small-cell lung cancer and ovarian carcinoma cells (25, 28). In contrast, Mori et al. (31) did not find stimulating effect on pancreatic cancer cells, including the cell line AsPC1, which we have also tested. This discrepancy could be attributable to different culture conditions: CXCL12-enhanced proliferation was better observed when suboptimal experimental conditions (i.e., no serum) are used.

An essential feature of metastatic cells is the ability to regulate their survival and to resist to apoptotic insults. The chemokine CXCL12 has been involved in promoting the survival of CD34+ hematopoietic progenitors, T lymphocytes (47–49), and of renal tumors and glioma cells (8, 26, 50). In this study, we first provide evidence that CXCL12 can protect CXCR4-positive pancreatic tumor cells from serum starvation-induced death or IL-1-induced damage, by decreasing the rate of apoptosis.

Overall, the results presented here show that metastatic pancreatic carcinoma cells express CXCR4 and that autocrine or paracrine loops centered on this chemokine receptor promote tumor cell migration, matrix degradation and invasion, proliferation, and survival. Therefore, the CXCL12-CXCR4 axis may represent a valuable therapeutic target in carcinoma of the pancreas.

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