Constitutive and Inducible Interleukin 8 Expression by Hypoxia and Acidosis Renders Human Pancreatic Cancer Cells More Tumorigenic and Metastatic

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ABSTRACT
The role and regulation of interleukin 8 (IL-8) in the growth and metastasis of SG, FG, and L3.3 variants derived from COLO 357 human pancreatic cancer cells were determined. After orthotopic implantation in the pancreas of nude mice, SG cells produced the smallest tumors, whereas L3.3 cells produced the largest tumors. SG cells produced no liver metastasis, whereas FG cells produced numerous liver metastases, and L3.3 cells produced more and larger liver metastases. In vitro analysis of IL-8 expression indicated that SG cells expressed the lowest level of IL-8 gene expression as determined by both Northern blot analysis and ELISA, whereas L3.3 cells expressed the highest level of IL-8. Immunohistochemical analysis of tumor lesions indicated that IL-8 overexpression was predominant in the regions surrounding necrotic areas, where cells were exposed to low oxygen tension (hypoxia) and acidic pH. In vitro treatment of FG tumor cells with hypoxia or acidosis led to an increased expression of IL-8. To directly determine the role of IL-8 in the growth and metastasis of pancreatic cancer, FG cells were transfected with IL-8 sense or antisense oligonucleotide expression vectors. The neo-resistance gene-transfected FG cells were used as controls. Decreased IL-8 expression after transfection with IL-8 antisense oligonucleotide expression vector retarded the growth of FG cells in mice after intrapancreatic implantation, which correlated with decreased tumor angiogenesis. Our data demonstrated that hypoxia and acidosis contribute to the overexpression of IL-8, which in turn plays an important role in tumor angio-

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3 The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; NF, nuclear factor.

INTRODUCTION
Pancreatic adenocarcinoma is presently the fifth leading cause of cancer death in the United States, with ~27,000 new cases and 25,000 cancer-related deaths per year (1). The incidence appears to be increasing. At the time of diagnosis, patients usually have locally advanced or metastatic disease to the lymph nodes, liver, lungs, and peritoneum (1, 2). Only ~10% of patients with pancreatic cancer are able to undergo a curative resection (1). The average survival from diagnosis to death is about 4–6 months, and the overall 5-year survival rate is <10%. Despite improvements in early diagnosis, surgical techniques, and chemotherapy, the majority of patients die because of the physiological effects of metastasis. The aggressive nature of this disease is related to several abnormalities in growth factors and their receptors. These abnormalities affect the downstream signal transduction pathways involved in the control of growth and differentiation. Other contributing molecular changes include mutation and inactivation of various oncogenes and tumor suppressor genes (1–3). In sum, these perturbations confer a tremendous growth advantage to pancreatic cancer cells.

Previous studies have focused on the role of growth factor families such as fibroblast growth factor, epidermal growth factor, transforming growth factor, and recently, insulin-like growth factor in pancreatic carcinogenesis (2). However, other growth factors and cytokines may also play an important role in the progression of human pancreatic cancer (4). Recent studies have shown that a variety of human tumor cells constitutively secrete the cytokine IL-8. In fact, IL-8 may promote the growth of bronchogenic carcinoma (5), non-small cell lung cancer (6), colorectal carcinoma (7), breast cancer (8), and ovarian cancer (9), and it may correlate with the metastatic potential of melanoma cells (10), ovarian cancer (9), prostate cancer (11), and gastric carcinomas (12). The promotion of tumor progression by IL-8 has been primarily explained by its ability to act as an autocrine growth factor for tumor cells (6, 13–16) as well as an angiogenic factor (5, 17–24). Whether IL-8 may also play a role in the biology and metastasis of human pancreatic cancer is unknown.

IL-8 belongs to the superfamily of CXC chemokines, has a wide range of proinflammatory effects, and was initially described as a neutrophil chemoattractant (25–28). IL-8 is produced by a wide range of cells including lymphocytes, mono-

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cytes, endothelial cells, fibroblasts, hepatocytes, and keratinocytes (25, 29). The production of IL-8 can be induced by various stimuli such as lipopolysaccharide, phorbol 12-myristate 13-acetate, IL-1, and TNF. It is not known whether the local tumor microenvironment, including oxygen deficiency and low extracellular pH, influence IL-8 expression, thereby contributing to the aggressiveness of human pancreatic cancer cells. As with many other solid tumors, growth and metastasis of human pancreatic cancer are dependent upon the development of an adequate vasculature (30). Despite the extensive angiogenesis, the overall vasculature in pancreatic tumors is poorly organized and marginally functional because of structural abnormalities. Consequently, the regional and temporal variations in tumor blood flow and tissue oxygen tension and diffusion contributions of hypoxic regions within solid tumors (31–34). A local decrease in oxygen tension and nutrient supply and low extracellular pH or acidosis have been considered to be major causes for the induction of many angiogenic molecular factors such as VEGF (31–34). Such factors profoundly influence many aspects of the tumor cell including metabolism, proliferation, metastasis, and therapeutic response (31–34).

In the present study, we assessed the IL-8 expression in human pancreatic cancer cell lines with different tumorigenic and metastatic potentials. The expression of IL-8 in vitro directly correlates with the increased tumorigenic and metastatic potential of pancreatic cancer cells. In vivo IL-8 was expressed predominantly in the tumor cells surrounding necrotic areas, where they are exposed to severe hypoxia and acidosis. In vitro exposure of pancreatic cancer cells to hypoxia and acidosis led to increased IL-8 expression. A decrease in IL-8 expression after transfection with IL-8 antisense oligonucleotide expression vector led to the suppression of tumor growth and metastasis in nude mice, whereas an increase in IL-8 expression after full-length IL-8 expression vector transfection resulted in decreased tumor growth and metastasis. Our data support the conclusion that constitutive and inducible overexpression of IL-8 by hypoxia and acidosis contributes to the aggressive biology of human pancreatic cancer.

**Materials and Methods**

**Cell Line and Culture Conditions.** SG, FG, and L3.3 variants were originally established from COLO 357 human pancreatic carcinoma cell lines by Vezeridis et al. (35). The cell lines were maintained in plastic flasks (5% CO_2:20% O_2:75% N_2 at 37°C) as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and vitamin solution (Flow Laboratories, Rockville, MD).

**Animals.** Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

**Analysis of IL-8 Gene Expression.** Human pancreatic carcinoma cells were treated as indicated. Cellular mRNA was extracted by using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA (2 μg) was separated electrophoretically on a 1.0% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon membrane (DuPont Co., Boston, MA) in 20× SSC, and UV-cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). The cDNA used in the study was a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8. The cDNA probes were labeled with [32P]dCTP using a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Equal loading of mRNA samples was monitored by hybridizing the same membrane filter with a human β-actin cDNA probe (9, 10). To measure IL-8 secretion, IL-8 levels in culture supernatants were determined by using an ELISA kit (Quantikine IL-8 ELISA kit; R&D Systems, Minneapolis, MN). This is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance versus the concentration of IL-8 in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, we determined the concentration of IL-8 in the unknown samples (9, 10).

**Treatment of Tumor Cells with Hypoxia and Acidosis in Vitro.** Tumor cells were seeded onto tissue culture dishes (15 cm) containing 20 ml of NaHCO_3-deficient RPMI 1640 (Sigma). The cells were allowed to recover for 24 h in a 5% CO_2:95% air incubator at 37°C. When the cells had reached 90% confluence, fresh medium was added. One set of plates was then transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) containing 5% CO_2:1% O_2:94% N_2 (hypoxia), and one set was transferred to an incubator containing 5% CO_2:20% O_2:75% N_2 (normoxia). For acidosis treatment, tumor cells were incubated in media of different pH levels for the indicated times in an incubator under normoxic conditions.

**Construction and Transfection of IL-8 Expression Vectors.** Human pancreatic cancer cell mRNA was isolated and reverse transcribed. The sequence spanning nucleotides 1–298 of the human IL-8 gene was amplified by PCR using forward primer 5’-ATGACTTCCAAGCTGGCCGTGGCT-3’ and reverse primer 5’-GAGAAGTGTGTGGAGGCGT-3’ (16). The PCR product was subcloned into the pcDNA3.1 expression vector downstream of cytomegalovirus promoter. Vectors with the IL-8 fragment in either sense (pcDNA-8S) or antisense (pcDNA-8A) orientations were sequenced and used for transfection experiments. To prepare tumor cells for plasmid transfection, FG tumor cells were plated in 10-cm dishes at 60% confluence. The monolayers were overlaid with a transfection solution containing 10 μg of pcDNA-8A, control pcDNA-8S, or pcDNA3.1 plasmids using a stable mammalian transfection kit from Stratagene. The cultures were placed in an incubator at 37°C for 12 h, washed, and then fed with fresh MEM. G418-resistant colonies were isolated and established in culture as individual cells lines. All of the lines were maintained in G418 medium and frozen after one to three in vitro passages. The expression of endogenous 1.8-kb IL-8 transcript in individual clones was determined by Northern blot analysis (9, 10).

**Tumor Growth and Metastasis.** For all in vivo experiments, tumor cells in exponential growth phase were harvested.
after a brief exposure to 0.25% trypsin:0.02% EDTA solution (w/v). The flask was tapped sharply to dislodge the cells, MEM was added, and the cell suspension was pipetted to obtain single-cell suspensions. The cells were washed, resuspended in Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS, and diluted to the desired cell number/inoculum. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >95% viability were used to determine tumorigenic and metastatic potential using orthotopic xenograft models. In brief, nude mice were anesthetized with methoxyflurane and placed in the supine position. An upper midline abdominal incision was made, and the pancreas was exteriorized. Tumor cells of 1 \(\times 10^6\) in 0.05 ml of HBSS were injected into the tail of the pancreas. Animals were killed 2 months after tumor inoculation, or when they became moribund. Tumors in the pancreas were harvested and weighed. Livers were fixed in Bouin’s solution for 24 h to differentiate the neoplastic lesions from the organ parenchyma, and metastases on the surface of liver were counted (double-blinded with the aid of a dissecting microscope as described previously (36).

**Immunohistochemistry.** Tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. The endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS for 12 min. The samples were incubated for 20 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated at 4°C in a 1:50 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA). The samples were then rinsed and incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were washed three times with distilled water, counterstained with Mayer’s hematoxylin (Bio-genex Laboratories, San Ramon, CA), and then washed once with distilled water and once with PBS. The slides were mounted with a Universal mount (Research Genetics) and examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Any brown-staining endothelial cell cluster distinct from adjacent microvessels, tumor cells, or other stromal cells was considered to be a single countable microvessel. Negative controls were done using nonspecific IgG. Areas containing the highest number of capillaries and small venules were identified by scanning tumor sections at low powers (×40 and ×100). After the areas (at least five/group) of vascular density were identified, individual vessels were counted in ×200 field (×20 objective and ×10 ocular (0.739-mm\(^2\)/field); Ref. 37). On the basis of the criteria described by Weidner et al. (37), vessel lumens were not required for a structure to be classified as a vessel. All vessel counts were performed on coded samples by two investigators.

**Statistics.** The significance of the data was determined by the Student’s t test (two-tailed). \(P < 0.05\) was deemed significant.

### RESULTS

**Expression of IL-8 in Human Pancreatic Cancer Cell Lines.** In the first set of experiments, the growth and metastasis of the COLO 357 human pancreatic cancer variants SG, FG, and L3.3 cells were determined. Tumor cells (1 \(\times 10^6\) cells/mouse) were injected into the pancreas of nude mice \((n = 5)\). Mice were killed on day 60 or when moribund. The tumors were collected and weighed, and liver metastases were counted with the aid of a dissecting microscope. Consistent with a previous report (35), SG cells were poorly tumorigenic and nonmetastatic, whereas FG cells were highly tumorigenic and produced many spontaneous liver metastases. Compared with FG cells, the L3.3 liver metastatic variant derived from FG cells produced larger primary pancreatic tumors and more and larger spontaneous liver metastases (Table 1). To determine IL-8 expression in the tumor variants differing in growth and metastasis, IL-8 mRNA expression in and protein secretion from SG, FG, and L3.3 cells were determined. The tumor cells were plated in 15-cm culture dishes at 75% confluence. Twenty-four h later, tumor cells were given fresh medium and incubated for 24 or 48 h. Culture supernatants were harvested for measurement of IL-8 production using

<table>
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<tr>
<th>Cell line</th>
<th>Primary tumor</th>
<th>Liver metastasis</th>
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<tr>
<td></td>
<td>Incidence</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>SG</td>
<td>5/5</td>
<td>0.22 ± 0.13</td>
</tr>
<tr>
<td>FG</td>
<td>5/5</td>
<td>0.98 ± 0.12(^a)</td>
</tr>
<tr>
<td>L3.3</td>
<td>5/5</td>
<td>1.61 ± 0.32(^b)</td>
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\(^a\) \(P < 0.05\) compared with SG cells.
\(^b\) \(P < 0.01\) compared with SG cells.
ELISA, and cellular mRNA was harvested for IL-8 mRNA analysis by Northern blotting. As shown in Fig. 1, all three variants expressed 1.8-kb IL-8 transcripts. The highest IL-8 expression was detected in L3.3 cells, and the lowest IL-8 expression was in SG cells (Fig. 1A). Consistent with mRNA expression, L3.3 cells secreted the highest amount of IL-8, whereas SG cells secreted 8-fold less (Fig. 1B). Therefore, we demonstrated a direct correlation between the expression of IL-8 in vitro and the tumorigenic and metastatic potential of these human pancreatic cancer cell lines.

Localization of IL-8 Expression in Human Pancreatic Cancer Cells Growing in the Pancreas of Nude Mice. To explore the possible mechanisms for IL-8-related tumor progression and the IL-8 expression pattern in human pancreatic cancer xenografts, FG tumor cells were orthotopically implanted into the pancreas of nude mice. Tumors were collected 60 days after implantation and processed for immunohistochemical analysis. IL-8 staining and H&E staining were performed on serial sections of the xenograft specimens. IL-8 protein expression was heterogeneous throughout the tumor lesion, and high IL-8 protein expression was predominantly observed in cells surrounding necrotic areas (Fig. 1C). Necrotic areas were identified by H&E staining (Fig. 1D). The cells surrounding necrotic areas are believed to undergo severe hypoxic and acidic stress (38, 39). Because mouse cells do not express the IL-8 gene, the IL-8 staining pattern suggested that a hypoxic and acidic environment may be one of the major factors inducing IL-8 overexpression from the xenografts.

Enhanced IL-8 Expression in Human Pancreatic Cancer Cells Exposed to Low Oxygen Tension and Acidic pH. To determine whether hypoxia induces IL-8 expression in human pancreatic carcinoma cells, FG human pancreatic cancer cells were incubated for 0, 2, 4, or 8 h under either normoxic or hypoxic conditions. To determine the effects of acidosis on IL-8 expression, FG cells were incubated in medium with pH of 7.35, 7.0, 6.8, 6.6, or 6.4 for 6 h. Cellular mRNA was isolated for determination of IL-8 expression by Northern blot analysis, and supernatants were collected for IL-8 secretion measurement by ELISA. We found that exposure of FG cells to hypoxia and acidosis resulted in a time-dependent increase in steady-state levels of IL-8 mRNA. IL-8 mRNA was increased after 2 h of hypoxia and continued to accumulate >5-fold by 8 h (Fig. 2, A and C). Consistent with the increased mRNA expression, the hypoxic and acidic FG cells released an increased amount of IL-8 protein into the culture medium in a time-dependent manner (Fig. 2, B and D). These data indicate that hypoxia and acidosis induce IL-8 expression in human pancreatic cancer cell lines.

Constitutive and Inducible IL-8 Expression in Human Pancreatic Cancer Cells Transfected with an IL-8 Antisense Expression Vector. The increased expression of IL-8 may lead to increased host lymphoid/myeloid cell infiltration because of its chemotactic properties and increased angiogenesis (5, 17–24). Therefore, blocking this process may lead to the suppression of human tumor progression. To that end, the parental FG tumor cells were transfected with pcDNA3.1 control
vector (N1 and N2, two individual clones), pcDNA-8Af IL-8 antisense oligonucleotide expression vector (A1, A2, and A3, three individual clones), or pcDNA-8Sf IL-8 sense oligonucleotide expression vector (S1, S2, and S3, three individual clones). All of these tumor cell lines were cultured in 100-mm dishes to 70% confluence. After a medium change (10 ml of MEM/100-mm dish), tumor cells were then cultured for 24 or 48 h. Cellular mRNA was extracted 24 h after medium change, and Northern blot analysis was performed. Culture supernatants were collected 24 and 48 h after medium change, and IL-8 protein in the supernatants was determined by ELISA and expressed as ng/ml. The IL-8 mRNA expression in parental, pcDNA3.1-transfected, pcDNA-8Sf-transfected, and pcDNA-8Af-transfected cells is shown in Fig. 3A. An 80–90% decrease in IL-8 mRNA expression was observed in the cells transfected with pcDNA-8Af (A1, A2, and A3), and there was no significant change in IL-8 expression in the parental FG cells and control cells transfected with pcDNA3.1 (N1 and N2) or pcDNA-8Sf (S1, S2, and S3). Consistent with decreases in IL-8 mRNA expression, the cells transfected with pcDNA-8Af (A1, A2, and A3) secreted a decreased amount of IL-8 protein into the culture supernatant (Fig. 3B). These data show that we could generate stable cell lines with decreased expression of IL-8 by transfection of an IL-8 antisense oligonucleotide expression vector.

To determine whether pcDNA-8Af1 transfection abrogated induction of IL-8 expression by hypoxia and acidosis, pcDNA-8Af-transfected (FG-N1) and pcDNA3.1-transfected (FG-A1) cells were incubated in vitro for 0–8 h at different extracellular pH for 6 h. Cellular mRNA was extracted, Northern blot analysis was performed using IL-8 cDNA probe, and equal loading of mRNA was monitored by hybridizing the same membrane filter with a β-actin cDNA probe (A and C). Quantitative analysis was performed by densitometry, standardized to β-actin, and expressed as fold difference from untreated controls (numbers in italic). Production of IL-8 protein by human pancreatic carcinoma cells was determined by ELISA (B and D). This is one representative experiment of two.

Growth and Metastasis of Human Pancreatic Cancer Cells Transfected with an IL-8 Antisense Expression Vector in Nude Mice. To determine tumorigenicity and metastatic potential of the above cell lines, parental FG cells, pcDNA3.1-transfected cells, pcDNA-8Af-transfected cells, and pcDNA-8Sf-transfected cells (1 × 10⁶ cells/mouse) were orthotopically injected into the pancreas of nude mice (n = 5). All control cells (parental FG-P, FG-N1, FG-N2, FG-S1, FG-S2, and FG-S3) produced large tumors, whereas the FG-A1, FG-A2, and FG-A3 cells did not (Table 2). The metastatic potential of the FG cells was determined 2 months after orthotopic implantation of tumor cells. Control FG-P, FG-N1, FG-N2, FG-S1, FG-S2, and FG-S3 cells produced numerous spontaneous liver metastases, whereas the FG-A1, FG-A2, and FG-A3 cells were nonmetastatic (Table 2). These data demonstrate that decreased expression of IL-8 leads to suppression of tumor formation and metastasis.

In Vitro Growth of Pancreatic Cancer Cells Transfected with an IL-8 Antisense Oligonucleotide Expression Vector. IL-8 has been shown previously to promote growth in various tumor cells (5–9). Therefore, the retarded growth of pcDNA-8Af-transfected cells may be attributable to decreased growth promotion by IL-8. To determine whether IL-8 is also an autocrine growth factor for human pancreatic cancer cells, the pa-
rental FG, control plasmid pcDNA3.1-transfected N1 and N2, pcDNA-8Af-transfected A1, A2, and A3, and pcDNA-8Sf-transfected S1, S2, and S3 tumor cells were seeded into 96-well plates (5 × 10^3 cells/well) for 48 h in the absence or presence of 10 ng/ml recombinant IL-8 or in the presence of 1 mg/ml IL-8 antibody. We chose the concentrations of IL-8 and IL-8 antibody that have been shown to have optimal effects in other tumor systems (6, 13–15, 40). Cell density was determined by methylthiotetrazole assay. The addition of IL-8 neutralizing antibody slowed the growth of all cells by up to 10%, but there was no statistical significance. The addition of exogenous recombinant IL-8 increased the growth of all cells by up to 20% (Fig. 5). These data suggest that IL-8 was a minimal autocrine growth factor for these human pancreatic cancer cell lines.

**Vessel Density of Human Pancreatic Tumors Growing in the Pancreas of Nude Mice.** Because the in vivo suppression of tumor growth after IL-8 antisense oligonucleotide expression transfection could not be completely explained by the autocrine effects of IL-8, other mechanisms must exist. Previous studies have indicated that IL-8 acts as a direct or an indirect angiogenic factor. To determine whether decreased IL-8 production led to angiogenesis suppression, the parental FG, control plasmid pcDNA3.1-transfected N1 or N2, pcDNA-8Af-transfected A1, A2, or A3, and pcDNA-8Sf-transfected S1, S2, or S3 tumor cells were injected into the pancreas of nude mice. Sixty days after injection, the tumors were removed and processed for immunohistochemical analyses of vascular formation. Vessels in these lesions were counted under light microscopy after immune staining of sections with anti-CD31 antibodies. As shown in Fig. 6, all control tumors (Fig. 6, A, C, and G) were highly vascularized. In contrast, the tumors formed by IL-8 antisense transfected cells (Fig. 6E) were less vascularized. By counting vessel number, there was a 53% decrease in vascular density of tumors expressing IL-8 antisense oligonucleotide compared with all control tumors. Consistent with the alteration of vascular density, high IL-8 expression was found in tumor lesions formed by control tumor cells (Fig. 6, B, D, and H), whereas low IL-8 expression was found in tumor lesions formed by FG-A1 cells (Fig. 6F).

**DISCUSSION**

IL-8 is a cytokine produced by many normal cells such as monocytes and fibroblasts as well as by several tumor cells (5–12). In this study, we showed that the human pancreatic cancer cell lines SG, FG, and L3.3 constitutively express IL-8.
expression vector (S1, S2, and S3, three individual clones) were injected into the pancreas of nude mice (pcDNA-8Af IL-8 antisense oligonucleotide expression vector (A1, A2, and A3, three individual clones), or pcDNA-8Sf IL-8 sense oligonucleotide expression vector (S1, S2, and S3, three individual clones) were injected into the pancreas of nude mice (n = 5). Mice were killed on day 60 or when moribund. Tumors were collected and weighed, and liver metastases were counted with the aid of a dissecting microscope.

A recent study by Luca et al. (40) indicated that IL-8 expression did correlate with experimental lung metastatic potential in a nude mouse model. Therefore, the relationship between IL-8 expression and metastatic ability is not certain. The discrepancy may, in part, be attributable to the use of different experimental methods and the variable requirement of IL-8 by different human tumor cell lines. In the present study, we used a tumor system consisting of variants originally derived from the same parental human pancreatic cancer cell line, and we used an orthotopic xenograft model to evaluate their tumorigenic and metastatic potential. We found that constitutive IL-8 expression in vitro directly correlates with the extent of local growth and production of spontaneous liver metastasis after implantation of these cancer cells into nude mice. Blocking IL-8 mRNA expression by transfection with an IL-8 antisense oligonucleotide expression vector decreased IL-8 protein secretion and suppressed tumor growth and metastasis in vivo. Our data clearly indicate that IL-8 plays a significant role in both local growth and metastasis in this pancreatic cancer model.

A large body of evidence suggests that overexpression of specific growth factors and their receptors may contribute to the biological aggressiveness of pancreatic cancer cells (1–3, 41). Since the first report showing that IL-8 acts as a growth factor (17), many recent studies have demonstrated that a variety of human tumor cells produce IL-8 and express the IL-8 receptor, thereby forming an autocrine growth stimulatory loop. However, the dependence on IL-8 for cell growth in vitro varied widely among different tumor types. Some tumor cell lines are apparently dependent upon IL-8, whereas others are not (13, 40). In our present study, we found that growth of FG tumor cells in vitro was minimally affected by the production of IL-8. This is based on our observation showing that a specific IL-8 neutralizing antibody only marginally blocks the growth of tumor cells in vitro. Even suppressing of IL-8 expression by 90% after transfection with pcDNA-8Af (the antisense oligonucleotide expression vector) affects the in vitro tumor growth rate by <10%. However, in vivo there was >90% suppression of tumorigenicity and 100% suppression of metastasis. Therefore, the role of IL-8 in the progression of pancreatic cancer is more complex than that of a simple autocrine growth stimulator.

Growth and metastasis of human pancreatic cancer depend upon its ability both to respond to its environment and to develop an adequate vasculature. In fact, to grow beyond the size of 2–3 mm in diameter, tumors must develop a new vascular bed (30, 34). This angiogenesis requires coupled interactions among malignant cells, cells resident in the tissue, and cells recruited from the circulation (30, 34). The induction of

### Table 2 Growth and metastasis of human pancreatic cancer cells transfected with control, sense, and antisense IL-8 oligonucleotide expression vector in the pancreas of nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incidence</th>
<th>Weight (g)</th>
<th>Incidence</th>
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<tr>
<td>FG-P</td>
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<td>0.85 ± 0.21</td>
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<td>0 (0–3)</td>
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<td>FG-N1</td>
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<td>0.94 ± 0.14</td>
<td>4/5</td>
<td>5 (0–13)</td>
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<tr>
<td>FG-N2</td>
<td>5/5</td>
<td>1.22 ± 0.21</td>
<td>3/5</td>
<td>1 (0–9)</td>
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<tr>
<td>FG-A1</td>
<td>2/5</td>
<td>0.21 ± 0.33</td>
<td>0/5*</td>
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<tr>
<td>FG-A2</td>
<td>3/5</td>
<td>0.34 ± 0.22</td>
<td>0/5*</td>
<td>0 (all 0)*</td>
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<tr>
<td>FG-A3</td>
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<td>0.12 ± 0.41</td>
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<tr>
<td>FG-S1</td>
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<td>FG-S2</td>
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<td>1.21 ± 0.26</td>
<td>3/5</td>
<td>6 (0–25)</td>
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<tr>
<td>FG-S3</td>
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<td>1.11 ± 0.37</td>
<td>3/5</td>
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* P < 0.01 compared with FG-P cells.

Schadendorf et al. (13) first demonstrated that IL-8 produced by human melanoma cells in tissue culture is an essential autocrine growth factor and is required for the growth of certain human melanoma cell lines. The expression of IL-8 apparently correlated with the growth of other tumors as well (6, 13–16), whereas the level of IL-8 expression in tumor cells did not correlate well with their ability to metastasize (13). However, a recent study by Luca et al. (40) indicated that IL-8 expression did correlate with experimental lung metastatic potential in a nude mouse model. Therefore, the relationship between IL-8 expression and metastatic ability is not certain. The discrepancy may, in part, be attributable to the use of different experimental methods and the variable requirement of IL-8 by different human tumor cell lines. In the present study, we used a tumor system consisting of variants originally derived from the same parental human pancreatic cancer cell line, and we used an orthotopic xenograft model to evaluate their tumorigenic and metastatic potential. We found that constitutive IL-8 expression in vitro directly correlates with the extent of local growth and production of spontaneous liver metastasis after implantation of these cancer cells into nude mice. Blocking IL-8 mRNA expression by transfection with an IL-8 antisense oligonucleotide expression vector decreased IL-8 protein secretion and suppressed tumor growth and metastasis in vivo. Our data clearly indicate that IL-8 plays a significant role in both local growth and metastasis in this pancreatic cancer model.

A large body of evidence suggests that overexpression of specific growth factors and their receptors may contribute to the biological aggressiveness of pancreatic cancer cells (1–3, 41). Since the first report showing that IL-8 acts as a growth factor (17), many recent studies have demonstrated that a variety of human tumor cells produce IL-8 and express the IL-8 receptor, thereby forming an autocrine growth stimulatory loop. However, the dependence on IL-8 for cell growth in vitro varied widely among different tumor types. Some tumor cell lines are apparently dependent upon IL-8, whereas others are not (13, 40). In our present study, we found that growth of FG tumor cells in vitro was minimally affected by the production of IL-8. This is based on our observation showing that a specific IL-8 neutralizing antibody only marginally blocks the growth of tumor cells in vitro. Even suppressing of IL-8 expression by 90% after transfection with pcDNA-8Af (the antisense oligonucleotide expression vector) affects the in vitro tumor growth rate by <10%. However, in vivo there was >90% suppression of tumorigenicity and 100% suppression of metastasis. Therefore, the role of IL-8 in the progression of pancreatic cancer is more complex than that of a simple autocrine growth stimulator.

Growth and metastasis of human pancreatic cancer depend upon its ability both to respond to its environment and to develop an adequate vasculature. In fact, to grow beyond the size of 2–3 mm in diameter, tumors must develop a new vascular bed (30, 34). This angiogenesis requires coupled interactions among malignant cells, cells resident in the tissue, and cells recruited from the circulation (30, 34). The induction of
angiogenesis is mediated by several molecules released by both tumor cells and host cells. Numerous molecules have been shown to contribute to the vascular formation of pancreatic cancer including fibroblast growth factors, epidermal growth factor, angiogenin, transforming growth factor, TNF, and VEGF (34). It is not known whether IL-8 plays an important role in pancreatic cancer angiogenesis, although several reports have indicated that IL-8 may play a role in other tumor types (5, 17–24). In the present study, we generated several stable cell lines expressing the IL-8 antisense oligonucleotide. All of the control tumors were highly vascularized and produced large tumors, whereas the cell line expressing IL-8 antisense oligonucleotide produced small tumors. The retarded tumor growth directly correlated with decreased blood vessel formation. In addition, transfection of FG tumor cells with a full-length sense IL-8 gene led to increased vessel formation and increased tumorigenic and metastatic potential (data not shown), which was very similar to that reported in data published previously (40). Therefore, IL-8 produced an important proangiogenic effect on human pancreatic cancer, which is consistent with the present evidence that IL-8 is an angiogenic factor (5, 17–24).

IL-8 is constitutively expressed in tumor cells in culture. However, the expression of IL-8 is still subject to regulation by various factors in vitro such as IL-1, TNF, and IFNs and in vivo by the tumor microenvironment (27, 29, 42). For example, melanoma cells growing in the subcutis of nude mice expressed higher levels of IL-8 than did those growing in the liver (42). It is well known that dynamically heterogeneous tumor blood flow and tissue oxygen tension are major components of the tumor microenvironment. The diffusion limitations and temporal and spatial changes in blood flow produce hypoxic regions in solid tumors. The increased anaerobic metabolism of hypoxic tumor

Fig. 6 Tumor vessel density and IL-8 expression in pancreatic cancer. The parental FG (A and B), control plasmid pcDNA3.1-transfected N1 (C and D), pcDNA-8Af-transfected A1 (E and F), or pcDNA-8Sf-transfected S1 (G and H) tumor cells were injected into the pancreas of nude mice. Sixty days later, the resulting tumors were removed and processed for immunohistochemical analysis. Vessels from these tumors were counted under a light microscope after immune staining of sections with anti-CD31 antibodies. IL-8 staining was performed as stated in Fig. 1 on the sections of FG (B), FG-N1 (D), FG-A1 (F), and FG-S1 (H) tumors. Note that significant decreased IL-8 expression and vessel formation in tumors were formed by FG-A1 cells as compared with all control tumors.
cells leads to elevated production of acidic metabolites, and the
reduced blood flow hinders removal of these metabolites, which
consequently leads to the accumulation of H\(^+\) and a decrease in
cellular pH. In the present study, we determined the patterns of
IL-8 expression in the human pancreatic tumor lesions growing
in the pancreas of nude mice. Although IL-8 protein was de-
tected in the tumor cells throughout the lesion, an elevated IL-8
expression was observed predominantly in tumor cells sur-
rounding the necrotic areas, where the tumor cells are believed
to undergo severe hypoxic and acidic stress (38, 39). Because
IL-8 can readily diffuse away from where they are produced, in
situ hybridization was then used to localize IL-8 mRNA, the
expression of which was consistent with the IL-8 immuno-
staining (data not shown). This IL-8 staining pattern suggests
that IL-8 expression may be further up-regulated by hypoxia and
acidosis, which is consistent with previous findings in astrocy-
toma and glioblastoma (38, 39). The up-regulation of IL-8
expression under conditions of hypoxia and acidosis was further
explored in vitro in FG human pancreatic carcinoma cells. We
found that exposure of FG cells to hypoxia and acidosis resulted in
a time-dependent increase in steady-state levels of IL-8
mRNA. Consistent with increased mRNA expression, hypoxic
FG cells released an increased amount of IL-8 protein into the
culture medium in a time-dependent manner. These data support
the hypothesis that hypoxia and acidosis induce IL-8 expression
in human pancreatic cancer cells. The increased expression of
IL-8 may lead to increased host lymphoid/myeloid cell infiltra-
tion attributable to its chemotactic properties and therefore
increased angiogenesis because of both direct and indirect an-
giogenic effects (5, 17–24). Consequently, hypoxia and acidosis
may contribute significantly to tumor angiogenesis, growth, and
metastasis in human pancreatic cancer, in part through its effect
on IL-8.

Regulation of IL-8 expression by IL-1, TNF, and glucocor-
ticoids has been well characterized (27, 43–45). However, the
mechanisms for hypoxia and acidosis-mediated up-regulation of
IL-8 remain unclear. The rapid induction and the subsequent
decline of IL-8 mRNA to baseline levels upon reoxygenation
(data not shown) is highly suggestive of transcriptional regula-
tion and posttranscriptional mRNA stabilization, which occurs
in many genes, including VEGF (46, 47). In fact, transcriptional
up-regulation of the VEGF gene during hypoxia is dependent
upon transcription by the transcription factor HIF-1 (46, 47),
which binds to an HIF-1 consensus site located in the 5’ flank-
ing region of the VEGF gene. Because no similar hypoxia-
responsive elements have been identified in the known 5’-flank-
atory regions of IL-8 gene, HIF-1 seems an unlikely
candidate to play a direct role in hypoxic regulation of IL-8 (27).
However, other potential regulatory sites are present within the
5’-flanking region. It has been documented that at −126 to
−120 bp, there is a putative AP-1 binding site, and the region from −94 to −71 bp shows sequence similarity with potential
binding sites for a NF-kB-like factor (−80 to −71 bp) and a
C/EBP-like factor NF-IL-6 (−94 to −81 bp). These binding
sites are contained in a minimal essential transcriptional regula-
tory element that confers responsiveness to IL-1, TNF-α, and
phorbol 12-myristate 13-acetate in a human fibrosarcoma cell
line (27). Recent studies have demonstrated that hypoxia activ-
ates both AP-1 and NF-kB (48–55). It is possible that AP-1-
and NF-kB-like factor-binding elements play a role in the in-
duction of the IL-8 gene in human pancreatic cancer cells during
hypoxia. Involvement of AP-1 and NF-kB in the process of
acidosis-mediated IL-8 induction also appears highly possible,
as suggested by several studies showing that extracellular pH
up-regulates genes including platelet-derived endothelial cell
growth factor/thymidine phosphorylase and the inducible iso-
form of nitric oxide synthase (56, 57). We are presently inves-
tigating the regulatory mechanisms of IL-8 gene expression in
this system by extracellular acidosis and hypoxia in human
tumor cells.

In summary, constitutive expression of IL-8 correlated with
the growth and metastasis of human pancreatic cancer cells
implanted in the pancreas of nude mice. Specific blockade of
IL-8 expression retarded the growth of human pancreatic cancer
cells after intrapancreatic implantation, which correlated with
decreased vascular formation. IL-8 may be expressed predomi-
nantly in the tumor cells experiencing hypoxia and acidosis in
the tumor lesions. In vitro exposure of pancreatic cancer cells to
hypoxia and acidosis did lead to increased IL-8 expression.
Collectively, these data suggest that the constitutive and induc-
able overexpression of IL-8 by hypoxia and acidosis contributes
to the aggressive biology of human pancreatic cancer.

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