Inhibition of Platelet-Derived Growth Factor Receptor Phosphorylation by STI571 (Gleevec) Reduces Growth and Metastasis of Human Pancreatic Carcinoma in an Orthotopic Nude Mouse Model

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ABSTRACT
Purpose: We evaluated the expression of platelet-derived growth factor (PDGF) ligands and receptors in clinical specimens of human pancreatic adenocarcinomas and determined the therapeutic effect of STI571 (Gleevec), a protein tyrosine kinase inhibitor of PDGF receptor (PDGFR), on human pancreatic carcinoma cells growing in the pancreas and liver of nude mice.

Experimental Design: Immunohistochemical staining for PDGF-AA and -BB ligands, PDGFR-α and -β, and phosphorylated PDGFR-α and -β was performed on 31 specimens of human pancreatic cancer and L3.6pl human pancreatic adenocarcinoma cell line. To determine the in vivo effects of STI571, nude mice with L3.6pl cells injected into the pancreas were randomized 7 days later to receive one of the following treatments: sterile water p.o. (control), STI571, gemcitabine, or a combination of STI571 and gemcitabine.

Results: In 29 of 31 clinical specimens of human pancreatic adenocarcinoma, both tumor cells and tumor-associated endothelial cells expressed phosphorylated PDGFR-α and -β. L3.6pl cells growing in culture expressed moderate amounts of PDGF-AA and little to no PDGFR-α or -β, whereas L3.6pl cells growing in the pancreas of nude mice expressed a high level of PDGF and receptors. Colocalization immunohistochemical analysis demonstrated expression of activated PDGFR-β by tumor-associated endothelial cells in both the pancreas and liver metastases. Tumors of mice treated for 4 weeks with STI571 (50 mg/kg or 100 mg/kg p.o. daily) were slightly smaller than controls. Tumors treated with gemcitabine and STI571 (50 mg/kg) were >70% smaller than tumors in control mice and 36% smaller than those in mice treated with gemcitabine only (P < 0.0002 and P < 0.04, respectively). Combination therapy also inhibited spontaneous metastasis to the liver. Tumors from mice treated with both STI571 and gemcitabine had decreased expression of activated (phosphorylated) PDGFR-α and -β, decreased mean vessel density, decreased cell proliferation, and increased apoptosis of tumor cells.

Conclusions: Collectively, these data show that activated PDGFR on tumor cells and tumor-endothelial cells can be a novel target for therapy of pancreatic carcinoma.

INTRODUCTION
Pancreatic adenocarcinoma remains one of the most aggressive cancers and is the fourth leading cause of cancer-related death in the United States (1). The general standard of care for pancreatic cancer is surgical resection and postoperative chemoradiation. Unfortunately, because of difficulties in diagnosis, only 10–20% of pancreatic cancers can be resected with curative intent at the time of diagnosis. Furthermore, most patients develop recurrent disease after surgical resection (1). Although gemcitabine was the first cytotoxic agent to show some efficacy in reducing symptoms and prolonging survival, the prognosis of patients with pancreatic adenocarcinoma remains dismal. Fewer than 5% of patients with pancreatic adenocarcinoma survive 5 years after the initial diagnosis, and the median survival duration is <6 months (2, 3).

Platelet-derived growth factor (PDGF) is a family of polypeptides that are important mitogens for many cell types (4). The A and B chains of PDGF combine to form AA, AB, and BB dimers that bind to protein tyrosine kinase receptors α and β [PDGF receptor (PDGFR)-α and PDGFR-β]. Once the PDGF receptors are thus activated, a variety of events can occur, including stimulation of cell growth, reorganization of actin, inhibition of gap junction communication, and inhibition of apoptosis (4). Dysregulation of these cellular events may result in tumorigenesis. Many tumor types, including gliomas and sarcomas, express PDGF and its receptors (reviewed in Ref. 4). Correlation between level of expression of PDGF-A and tumor grade of human gliomas provides indirect evidence that PDGF is involved in tumor progression (5). Moreover, antagonists to PDGF can inhibit growth of human glioma cells (6, 7).

The first evidence of PDGF expression in pancreatic cancer was provided by Ebert et al. (8). PDGF-A and -B chains and
PDGFR-β transcripts were identified in the human pancreatic cancer cell lines PANC-1 and HPAF. Thirteen samples of human pancreatic cancers were examined for expression of PDGF-A and β and their receptors. PDGF-A was present in all of the specimens at levels equal to those of normal pancreatic tissue, although PDGF-B was found in low levels in only 6 of the 13 samples. The tumor samples also exhibited a significant 7-fold increase in PDGFR-α and PDGFR-β mRNA levels compared with samples of normal pancreas (8).

Compounds that inhibit PDGFR kinases have been developed recently. STI571 (Gleevec, also known as imatinib mesylate; Novartis Pharma, Basel, Switzerland) is a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class that was developed initially for its selectivity against the BCR-ABL fusion protein present in nearly all patients with chronic myeloid leukemia (9). In addition, STI571 was found to inhibit the kinase activity of the PDGFR-α and -β as well as c-Kit (10). Clinical studies have demonstrated the safety and efficacy of STI571 for treatment of chronic myelogenous leukemia and c-Kit-positive gastrointestinal stromal tumors (11, 12). In an animal model of dermatofibrosarcoma protuberans, STI571 was shown to inhibit tumor growth by blocking PDGFR and induction of apoptosis (13). In preclinical studies from our laboratory, STI571 was shown to inhibit tumor growth by blocking PDGFR and induction of apoptosis (13). In preclinical studies from our laboratory, STI571 was shown to inhibit the growth of human prostate cancer metastases to bone (14), as well as osteosarcoma tumors (15), through blockade of PDGF-mediated signaling. To date, the effect of targeted therapy directed at inhibiting PDGFR activity in pancreatic cancer has not been reported. The current study, therefore, examines the presence of PDGF-A and -B, and phosphorylated PDGFR-α and -β in human pancreatic adenocarcinomas as potential therapeutic targets for STI571. We then evaluated the effectiveness of oral STI571 therapy in orthotopic nude mouse model of pancreatic cancer both as single-agent therapy and as combination therapy with gemcitabine.

MATERIALS AND METHODS

Pancreatic Cancer Cell Lines and Culture Conditions. The human metastatic pancreatic cancer cell line L3.6pl (16) was maintained in DMEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, 1-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO2 and 95% air. The cultures were free of Mycoplasma and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler’s encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Reagents. STI571 (Gleevec, Novartis) was stored at room temperature and diluted in sterile water as necessary at the time of use. Gemcitabine (Gemzar, Eli Lilly Co., Indianapolis, IN) was kept at room temperature and dissolved in 0.9% NaCl on the day of use. All of the reagents used in tissue culture were free of endotoxin as determined by the Limulus amebocyte lysate assay (sensitivity limit of 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA). Antibodies were purchased as follows: polyclonal rabbit anti-PDGFR-α, polyclonal rabbit anti-PDGFR-β, polyclonal goat antiphosphorylated PDGFR-α, polyclonal goat antiphosphorylated PDGFR-β, polyclonal rabbit anti-PDGFR-A, and polyclonal rabbit anti-PDGFR-B (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA); peroxidase-conjugated affinity pure F(ab′)2 goat antirabbit IgG F(ab′)2 (Jackson Research Laboratories, West Grove, CA); monoclonal rat antimouse CD31/PECAM-1 (PharMingen International, San Diego, CA); peroxidase-conjugated goat antirat IgG (H+L); rabbit antigoat IgG F(ab′)2 fragment (Jackson Research Laboratories); polyclonal rabbit anti-human c-kit (CD117; Dako, Carpenteria, CA); antigoat biotinylated secondary antibody (Biocare); streptavidin horseradish peroxidase; mouse IgG1 antihuman CD34 (Biogenex, San Ramon, CA); streptavidin Alexa Fluor 594-conjugated antibody and streptavidin Alexa Fluor 488-conjugated antibody (Molecular Probes, Eugene, OR); and Biogenex multilink and Biogenex label used for enhancing detection of antibodies (Biogenex).

Other reagents used for immunohistochemical (IHC) analysis include Hoescht Dye 3342 (MW 615.9) (Hoechst, Los Angeles, CA), fish gel (Cold Water Fish Skin Gelatin, 40% Aurion; Electron Microscopy Sciences, Fort Washington, PA), Gill’s hematoxylin from Sigma Chemical Co. (St. Louis, MO), and pepsin from Biomeda (Foster City, CA).

Tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), M2128] was purchased from Sigma Chemical Co., and a stock solution was prepared by dissolving 5 mg of MTT in 1 ml PBS and filtering the solution to remove particulates. The solution was protected from light and stored at 4°C. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) assay was performed by using a commercial Apoptosis Detection kit (Promega, Madison, WI) with modifications.

In Vitro Cytotoxicity Assay. Tumor cells (1 × 10³) were seeded into 38-mm² wells of flat-bottomed 96-well plates in triplicate and allowed to adhere overnight. The spent medium was then removed and the cultures were refed with new medium (negative control) or medium containing different concentrations of STI571. After 3 days (control cultures did not reach confluence), the number of metabolically active cells was determined by the MTT assay (17). After a 2–4-h incubation period in medium containing 0.42 mg/ml of MTT, the cells were subjected to lysis in DMSO. An MR-500 96-well microtiter plate reader at 570 nm (Dynatech, Inc., Chantilly, VA) monitored the conversion of MTT to formazan by metabolically active cells. Growth inhibition was calculated from the following formula:

\[
\text{Cytostasis} \% = \left[1 - \frac{A}{B}\right] \times 100
\]

where A is the absorbance of treated cells and B is the absorbance of control cells.

Western Blot Analysis. L3.6pl pancreatic adenocarcinoma and MG63 osteosarcoma cells were grown in MEM containing 10% fetal bovine serum, washed, scraped into PBS
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Snap-frozen in liquid nitrogen, and stored at -70°C. Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were incubated with the primary antibody overnight at 4°C. Visible liver metastases were counted with the aid of a dissecting microscope, and the tissues were processed for H&E staining. All of the macroscopically enlarged regional (celiac and para-aortal) lymph nodes were harvested, and the presence of metastatic disease was confirmed by histological review.

**IHC Analysis to Detect PDGF-A, PDGF-B, PDGFR-α, PDGFR-β, Phosphorylated PDGFR-α, and Phosphorylated PDGFR-β.** After orthotopic injection of L3.6pl human pancreatic adenocarcinoma cells into nude mice, tumors were allowed to grow for 3 weeks. Paraffin-embedded tumors were then stained for the presence of PDGF-A and -B ligands and PDGFR-α and -β receptors. Sections (4–6 μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, treated with a graded series of alcohols [100%, 95%, and 80% ethanol/ddH2O (v/v)], and rehydrated in PBS (pH 7.5). Slides were fixed in cold acetone, in acetone/chloroform (1:1), and again in cold acetone. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. No antigen retrieval was performed. Samples were exposed to protein block (5% normal horse serum and 1% normal goat serum in PBS) and incubated with each primary antibody at the appropriate dilution overnight at 4°C. After incubation with peroxidase-conjugated goat antirabbit IgG for 1 h at room temperature, a positive reaction was detected by exposure to stable 3,3′-diaminobenzidine for 10–20 min. Slides were counterstained with Gill’s hematoxylin.

Pancreatic tumors harvested from mice in the therapy experiments were fixed in formalin and embedded in paraffin. The paraffin-embedded tumors were stained for PDGF-AA and PDGFR-α as described previously. Sections were also stained for phosphorylated PDGFR-α (p-PDGFR-α) and -β as follows: slides were deparaffinized and hydrated with PBS as described earlier, incubated with 3% hydrogen peroxide in PBS, treated with protein block for 15 min (4% fish gel in PBS), and incubated with the primary antibody overnight at 4°C. Biotiny-
lated antigoat secondary antibody was added for 30 min, followed by three washes in PBS, and streptavidin horseradish peroxidase diluted in house detection solution was added for 30 min. A positive signal was detected by using 3,3′-diaminobenzidine.

Analysis of PDGF Expression in Human Pancreatic Adenocarcinomas. Clinical specimens of 36 human pancreatic carcinomas were examined. Five human pancreatic tumor specimens were evaluated for the presence of PDGF-AA, PDGF-BB, PDGF-α, and PDGF-β. The other 31 human pancreatic cancer specimens were evaluated for the presence of activated PDGF-α and PDGF-β. All of the pancreatic adenocarcinoma samples were obtained from surgical specimens resected from patients at The University of Texas M. D. Anderson Cancer Center who did not receive neoadjuvant treatment. Nonmalignant pancreatic tissue obtained from 10 patients undergoing surgery for benign conditions was designated as “normal” pancreas. Both pancreatic tumors and normal pancreas tissue were histologically confirmed by an experienced pathologist. Paraffin-embedded sections were then subjected to IHC staining for PDGF-A, PDGF-B, PDGF-α, PDGF-β, p-PDGFR-α, and p-PDGFR-β as already described. Samples were also stained for c-kit by using polyclonal rabbit antihuman c-Kit and goat antirabbit horseradish peroxidase IgG F(ab′)2 fragment as secondary antibody.

IHC Determination of CD31/PECAM-1 (Endothelial Cells), Proliferating Cell Nuclear Antigen (PCNA), and TUNEL. Paraffin-embedded tissues were used for IHC identification of PCNA. Sections were deparaffinized and rehydrated in PBS as described previously, microwaved for 5 min for “antigen retrieval,” incubated at 4°C with the primary antibody overnight (mouse IgG2a anti-PCNA), and incubated for 1 h at room temperature with a secondary antibody (peroxidase-conjugated rat antimouse IgG2a). Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), in acetone/chloroform (v/v; 5 min), and again in acetone (5 min) and washed with PBS. IHC procedures were performed as described previously (18). Positive reactions were visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10–20 min. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 30 s, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. For the quantification of mean vessel density (MVD) in sections stained for CD31, 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified according to the method described previously (19, 20). For quantification of PCNA expression, the number of positive cells was counted in 10 random 0.159-mm² fields at ×100 magnification.

Analysis of pancreatic tumors for apoptotic cells was performed by using a commercially available TUNEL kit with the following modifications: samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton-X 100 for 15 min at room temperature. After being washed with PBS, the samples were incubated with equilibration buffer for 10 min at room temperature, and reaction buffer was added (containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme). Samples were incubated in a humidified chamber at 37°C for 1 h in the dark and immersed in 2× SSC for 15 min to terminate the reaction. Immunofluorescence microscopy was performed by using a ×40 objective (Zeiss Plan/Neofluor) on an epifluorescence microscope equipped with narrow band-pass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY). Images were captured by using a chilled cooled charge coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were additionally processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). To quantify the apoptotic events, the number of cells undergoing apoptosis was counted in 10 random 0.159-mm² fields at ×100 magnification and expressed as a percentage of the total number of tumor cells in the same field.

Double Immunofluorescence Staining for CD34/PECAM-1 (Endothelial Cells) and p-PDGFR-β. Frozen specimens from human pancreatic adenocarcinomas and pancreatic adenocarcinoma liver metastases were sectioned (8–10 μm), mounted on positively charged slides, air-dried for 30 min, and fixed in cold acetone, in acetone/chloroform (1:1), and again in acetone for 5 min each. Samples were washed with PBS and incubated with protein-blocking solution containing 4% fish gel in PBS for 20 min at room temperature. Primary antibody (mouse IgG1 antihuman CD34) was added to the specimens and allowed to incubate at 4°C overnight. Samples were washed in PBS, blocked with 4% fish gel for 5 min, and incubated first with the Biogenex link agent for 30 min and then with streptavidin-Alexa 594 secondary antibody for 30 min. Samples were again washed in PBS, blocked briefly in 4% fish gel, and incubated with

![Fig. 1](Image) Western blot. Protein lysates were obtained from L3.6pl pancreatic adenocarcinoma and MG63 osteosarcoma cells (positive control). Proteins were extracted from L3.6pl pancreatic tumors grown in nude mice for 3 weeks and from normal pancreas tissue in nude mice. Samples were analyzed for expression of platelet-derived growth factor (PDGF)-AA, PDGF-BB, PDGF-α, and PDGF-β.
antibody against human p-PDGFR-β at 4°C overnight. After three washes in PBS, samples were blocked briefly with 4% fish gel and incubated first with the secondary biotinylated mouse antigoat antibody for 30 min and then with the streptavidin-Alexa 488 antibody for 30 min. Samples were briefly incubated with Hoechst stain to visualize the nuclei. Endothelial cells were identified by red fluorescence, and p-PDGFR-β was identified by green fluorescence. The presence of p-PDGFR-β in tumor endothelial cells was detected by colocalization of red and green fluorescence, which appeared yellow. Three liver metastasis specimens obtained from patients with primary pancreatic adenocarcinomas were analyzed in the same manner for the presence of p-PDGFR-β in endothelial cells.

**Fig. 2** Immunohistochemical analysis of platelet-derived growth factor (PDGF) expression in human pancreatic tissues. Specimens of tumors and nonmalignant pancreas tissue from patients with pancreatic adenocarcinoma (untreated) were sectioned, and sections were immunostained for expression of PDGF-AA and -BB ligands, PDGFR-α and PDGFR-β, and phosphorylated PDGFR-α and PDGFR-β. Pancreatic adenocarcinoma specimens stained weakly for PDGF-AA and PDGF-BB, and more strongly for PDGFR-α and PDGFR-β. Phosphorylated PDGFR-α and -β were strongly expressed in tumor specimens. Normal (nonmalignant) pancreas tissue was negative for p-PDGFR-α and -β.
Statistical Analysis. The volumes of the pancreatic tumors, body weights, and quantifications of PCNA, TUNEL, and CD31 were compared by the unpaired Student t test.

RESULTS

In Vitro Effect of STI571 on Human Pancreatic Cancer Cells. To determine the antiproliferative activity of STI571, L3.6pl human pancreatic cancer cells were cultured for 3 days in medium containing increasing concentrations of STI571 (0–20 μM). The MTT assay revealed that the IC<sub>50</sub> was 17 μM (data not shown).

Expression of PDGF and PDGF-R in Pancreatic Cancer Cells. Western blotting using antibodies specific for PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β was performed to determine the expression of PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β in L3.6pl human pancreatic adenocarcinoma cells growing in culture or in the pancreases of nude mice. Cultured L3.6pl cells expressed PDGF-A (compared with MG63 osteosarcoma cells used as a positive control). Expression of PDGFR-α, PDGF-B, and PDGFR-β was barely detectable (Fig. 1). In contrast, L3.6pl cells injected into the pancreases of nude mice expressed similar levels of PDGF-A as L3.6pl cells grown in vitro but much higher levels of PDGFR-α and PDGFR-β. Normal pancreas from nude mice had similar patterns of expression of PDGF and PDGFR as L3.6pl cells growing in vitro.

IHC Analysis of Clinical Specimens of Human Pancreatic Cancer. Five human pancreatic adenocarcinoma samples were analyzed for expression of PDGF-AA, PDGF-BB, PDGFR-α, and PDGFR-β. All five of the samples were positive for all four of the proteins (Fig. 2). In addition, 31 clinical specimens of pancreatic carcinomas were evaluated for expression of p-PDGFR-α and p-PDGFR-β; 28 (90.3%) stained positive for p-PDGFR-α and 29 (93.5%) for p-PDGFR-β. Strong PDGF immunoreactivity was evident in the cytoplasm of both ductal-like cancer cells and poorly differentiated cancer cells (Fig. 2). Several tumor samples had faint staining of fibroblasts in the stroma. In contrast, only 1 of 10 nonmalignant pancreas...
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Tissues were prepared for immunohistochemical analysis as described in

biweekly i.p. injections of gemcitabine (15 mg/kg) or water (control). The mice were treated for 4 weeks and then killed and subjected to necropsy.

treated with biweekly i.p. injections of gemcitabine (15 mg/kg) alone, daily oral STI571 (50 mg/kg) alone, combined daily oral STI571 (50 mg/kg)

samples was positive for p-PDGFR-α, and 3 of 10 were positive for p-PDGFR-β. Weak immunoreactivity for p-PDGFR-α and p-PDGFR-β was seen only in the cytoplasm of islet cells. When specimens containing both pancreatic adenocarcinoma and adjacent nonmalignant pancreas tissue were analyzed, ductal-like cancer cells exhibited strong staining for both p-PDGFR-α and p-PDGFR-β (Fig. 2). The surrounding desmoplastic tissue also exhibited weak to moderate immunoreactivity, whereas immunoreactivity in adjacent nonmalignant pancreas was weak or absent.

Using a double immunofluorescence staining technique, we examined whether endothelial cells within the human pancreatic adenocarcinoma specimens expressed p-PDGFR-β (Fig. 3). Immunoreactivity for CD34 (endothelial cells) is shown in red, and that for p-PDGFR-β in green. When colocalized, endothelial cells that express p-PDGFR-β appeared yellow. As shown in Fig. 3, both primary pancreatic tumors and liver metastases from pancreatic adenocarcinomas demonstrated colocalization of CD34 and p-PDGFR-β, indicating the presence of p-PDGFR-β on endothelial cells in primary and metastatic pancreatic tumor specimens.

Effect of STI571 on Human Pancreatic Cancer in an Orthotopic Nude Mouse Model. We have described previously an orthotopic nude mouse model of human pancreatic cancer (16). In the present study, L3.6pl human pancreatic adenocarcinoma cells were injected into the pancreases of athymic nude mice. Seven days later, the mice were randomized into six groups of 10 mice each. The control group received sterile water daily by p.o. gavage, the second group daily STI571 50 mg/kg p.o., the third group daily STI571 100 mg/kg p.o., the fourth group twice weekly i.p. gemcitabine 15 mg/kg, the fifth group i.p. gemcitabine and daily STI571 50 mg/kg p.o., and the sixth group i.p. gemcitabine and daily STI571 100 mg/kg p.o. The mice were treated for 4 weeks. On day 35, the mice were killed and subjected to necropsy. Incidence of tumor formation was 100% in all of the groups. As shown in Table 1, treatment with neither STI571 nor gemcitabine alone significantly affected body weight as compared with the control group. Mice treated with STI571 had smaller tumors than control mice, but the difference was not statistically significant. Mice treated with both STI571 and gemcitabine had significantly smaller tumors than the control group. Mice treated with the lower dose of STI571 (50 mg/kg) in combination with gemcitabine had tumors that were significantly smaller (323.0 mm³; P < 0.0002) than those in control mice (1101.6 mm³) or in mice treated with gemcitabine alone (503.8 mm³; P < 0.04). Mice treated with the higher dose of STI571 (100 mg/kg) in combination with gemcitabine had a median tumor volume (448.4 mm³) that was significantly smaller than that of control mice, but not significantly different from that of mice treated with gemcitabine alone (P = 0.13).

To determine the biological effects of treatment with STI571 and gemcitabine, pancreatic tumors were harvested from mice from the different treatment groups and processed as either frozen sections or formalin-fixed specimens for IHC staining. Tumors from mice treated with the most biologically effective dose of STI571 (50 mg/kg), as well as tumors from control mice and mice treated with gemcitabine only, were analyzed for expression of PDGF-A, PDGFR-α, p-PDGFR-α, and p-PDGFR-β. Tumors from mice treated with STI571, gemcitabine, or a combination of STI571 and gemcitabine expressed similar levels of PDGF-AA ligand as tumors from control mice (Fig. 4). Tumors from all of the treatment groups had a similar pattern of PDGFR-α expression. However, expression of p-PDGFR-α was much lower in tumors from mice treated with either STI571 alone or STI571 combined with gemcitabine than in control tumors (Fig. 4).

MVD in the tumors was measured by IHC staining with antibodies against CD31 (Fig. 5; Table 2). Tumors treated with STI571 alone or STI571 combined with gemcitabine had a significantly lower MVD than control tumors (18.3 ± 4.2 and 22.0 ± 6.6 versus 40.8 ± 6.6; P < 0.0001). Furthermore, the MVD in tumors from mice treated with STI571 differed significantly from that of tumors from mice treated with gemcitabine alone (39.0 ± 5.0; P > 0.001). There was no difference in MVD between the control group and the gemcitabine-only group (P = 0.64).

The effect of STI571 treatment on apoptosis in the pancre-

<table>
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<th>Treatment group</th>
<th>Body Weight Median</th>
<th>Range</th>
<th>Tumor incidence</th>
<th>Tumor volume (mm³) Median</th>
<th>Range</th>
<th>Tumor weight (g) Median</th>
<th>Incidence</th>
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<td>18.6–29.8</td>
<td>10/10</td>
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Table 1: Antitumor effects of STI571 and gemcitabine in L3.6pl pancreatic tumors

* L3.6pl human pancreatic cancer cells (1 × 10⁶) were injected into the pancreases of nude mice. Seven days later, different groups of mice were treated with biweekly i.p. injections of gemcitabine (15 mg/kg) alone, daily oral STI571 (50 mg/kg) alone, combined daily oral STI571 (50 mg/kg) and biweekly i.p. injections of gemcitabine (15 mg/kg) or water (control). The mice were treated for 4 weeks and then killed and subjected to necropsy. Tissues were prepared for immunohistochemical analysis as described in “Materials and Methods.”

P < 0.0002 versus control.

P < 0.04 versus STI571 alone.

P < 0.01 versus control or gemcitabine alone.
atic tumors was evaluated by using the TUNEL method (Fig. 5; Table 2). In control tumors, the mean percentage of apoptotic tumor cells was 3.6% ± 0.5. The percentage of apoptotic cells was greater in tumors from mice treated with STI571 alone or STI571 combined with gemcitabine (16.8% ± 4.6 and 73.2% ± 2.5, \( P \leq 0.05 \)) than in those from control mice or mice treated with gemcitabine alone. Treatment with STI571 as a single agent also resulted in significantly higher incidence of apoptotic tumor cells than treatment with gemcitabine alone (16.8% ± 4.6 versus 3.0% ± 0.2; \( P = 0.05 \)). There was no difference in the percentage of apoptotic cells between tumors in the control group and mice treated with gemcitabine alone (3.6% ± 0.5 versus 3.0% ± 0.2; \( P = 0.24 \)).

The pancreatic tumors were evaluated for cell proliferation by IHC staining for PCNA (Fig. 5; Table 2). In the control group, the mean number of PCNA-positive cells was 343 ± 45. After 28 days of treatment with STI571 at 50 mg/kg, treated tumors contained significantly fewer PCNA-positive cells than controls (282 ± 45; \( P < 0.01 \)). Tumors treated with gemcitabine alone exhibited fewer PCNA-positive cells than either the control group or the STI571 group (195 ± 67; \( P < 0.01 \) versus control; \( P < 0.05 \) versus STI571). The combination of STI571
and gemcitabine resulted in the lowest number of PCNA-positive tumor cells (152 ± 30), which was significantly different from the number in the control group (P < 0.01) but not from the number in the gemcitabine-only group (P = 0.07).

**DISCUSSION**

The studies reported here reveal that L3.6pl human pancreatic cancer cells growing in culture express the PDGF-A ligand at higher levels than the PDGF-B ligand. Western blot analysis demonstrated that the cells express low levels of PDGFR-α and PDGFR-β. When these cells are injected into the pancreases of nude mice in an orthotopic model of pancreatic cancer, protein lysates from the resulting tumors express even higher levels of PDGFR-α and -β. Furthermore, when these pancreatic tumors are sectioned and analyzed immunohistochemically, both PDGF-A and -B ligands, and both PDGFR-α and -β receptors are strongly expressed, and the receptors are phosphorylated.

IHC staining of 5 clinical specimens of human pancreatic adenocarcinoma revealed that all of the samples were positive for expression of PDGF-AA, PDGF-BB, PDGFR-α, and PDGFR-β. Examination of 31 additional tumors determined that the activated or phosphorylated PDGFR-α and -β were present in 90–93.5% (28 and 29 of 31 specimens, respectively). In contrast, only 10–30% of nonmalignant (“normal”) human pancreas samples were positive for p-PDGFR-α or p-PDGFR-β. The weakly positive staining was present mostly in the cytoplasm of islet cells. The higher levels of the receptors α and β

![Fig. 5 Immunohistochemical analysis for mean vessel density, cell proliferation, and apoptosis in L3.6pl pancreatic tumors in mice treated with STI571 and/or gemcitabine. After 4 weeks of treatment, pancreatic tumors were harvested and sections stained with anti-CD31 or antiproliferating cell nuclear antigen antibodies or analyzed for apoptosis by using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling assay.]
Table 2  Immunohistochemical analysis of endothelial cells, dividing cells, and apoptotic cells after treatment with STI571 and gemcitabine

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>CD31-positive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Termianl deoxynucleotidyl transferase-mediated dUPT-biotin end labeling (TUNEL) positive&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Proliferating cell nuclear antigen (PCNA) positive&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>40.80 ± 6.61</td>
<td>3.58 ± 0.49</td>
<td>343 ± 45</td>
</tr>
<tr>
<td>STI571 (50 mg/kg)</td>
<td>18.33 ± 4.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.77 ± 4.57&lt;sup&gt;e&lt;/sup&gt;</td>
<td>282 ± 45</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>39.00 ± 5.00</td>
<td>2.96 ± 0.21</td>
<td>195 ± 67&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gemcitabine + STI571 (50 mg/kg)</td>
<td>22.00 ± 6.56&lt;sup&gt;e&lt;/sup&gt;</td>
<td>73.23 ± 2.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>152 ± 30&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number (mean ± SD) positive cells/field determined by measuring 10 random fields (0.159 mm<sup>2</sup>) at ×100 magnification.
<sup>b</sup> Percentage of TUNEL-positive tumor cells (out of total number of cells) per 0.159-mm<sup>2</sup> field at ×100 magnification.
<sup>c</sup> Number (mean ± SD) of PCNA-positive tumor cells/field determined by measuring 10 random 0.159-mm<sup>2</sup> fields at ×100 magnification.
<sup>d</sup> P < 0.05 versus control.
<sup>e</sup> P < 0.01 versus control.
<sup>f</sup> P < 0.05 versus STI571.
<sup>g</sup> P = 0.001 versus control or gemcitabine alone.

...in the lysates from pancreatic tumors grown in nude mice than in cultured L3.6pl cells imply that in vivo mechanisms may be responsible for an autocrine loop in pancreatic tumors. These findings are similar to those reported by Ebert et al. (8), who demonstrated that PDGF-A and -B chains and PDGFR-β are present in PANC-1 and HPAF human pancreatic cancer cell lines. In human pancreatic cancer tissues, PDGF-A was abundant by Northern blotting, and ~50% of the specimens exhibited the PDGF-B chain. Both receptors α and β were present in the pancreatic cancer tissue specimens at 7-fold higher levels than in normal pancreas specimens. Our data showing the presence of PDGF receptors in fibroblasts in the stromal tissue surrounding the pancreatic cancer agree with a previous report (8). Others have also reported the expression of PDGFs in stromal tissue surrounding cancers such as breast carcinoma, colorectal cancer, and small-cell lung carcinoma (reviewed in Ref. 4). Thus, the presence of PDGF and its receptors in connective tissue associated with cancers may contribute to a paracrine loop resulting in up-regulation of PDGF expression, and growth stimulation, of the tumor cells. Our present study demonstrates overexpression of the activated forms of the PDGFR-α and -β in the majority of human pancreatic cancer samples that we analyzed. This finding suggests that blockade of PDGFR-dependent growth pathways may be an effective strategy to inhibit growth of pancreatic adenocarcinomas.

Blockade of PDGFR phosphorylation by STI571 inhibited proliferation of L3.6pl cells. After 3 days of incubation with the drug, the IC<sub>50</sub> was 17 μM. We have reported previously that growth of MG63 osteosarcoma cells, when stimulated by PDGF-BB, was significantly inhibited by lower concentrations of STI571 (1.0 and 10.0 μM; Ref. 15). The discrepancy in sensitivity is likely due to the different levels of expression of PDGFR by the two cell lines.

Although a relatively high dose of STI571 was required for inhibition of growth of L3.6pl pancreatic cancer cells under in vitro conditions, STI571 was more potent in inhibiting growth of cells implanted into the pancreases of nude mice. The most effective treatment was the combination of STI571 50 mg/kg p.o. daily and gemcitabine 15 mg/kg i.p. twice weekly. After 4 weeks, mice that received this treatment had significantly smaller (71% smaller) pancreatic tumors than control mice. Tumors in the combination treatment group were 36% smaller than those in the gemcitabine-only group. Tumors in the STI571-only group were 23% (50 mg/kg) or 30% (100 mg/kg) smaller than tumors in the control group. These differences, however, were not statistically significant. The incidence of liver and peritoneal metastases was also lower in the mice that received combination therapy than in control mice. Neither STI571 nor gemcitabine, nor the combination of the two drugs significantly affected body weight of the mice. The most effective treatment in this orthotopic nude mouse model of pancreatic cancer was the combination of STI571 50 mg/kg daily and gemcitabine. This therapeutic regimen inhibited pancreatic tumor growth to a greater degree than either drug alone. Similarly, this combination yielded a higher incidence of apoptosis, a lower proliferation rate as measured by PCNA staining, and lower MVD than either drug alone.

These findings agree with a recent report from our laboratory on therapy of human prostate cancer bone metastases showing that STI571 was more effective in producing a therapeutic effect when combined with paclitaxel than when given alone (15). Several possible mechanisms could underlie the increased efficacy of the combination of STI571 and gemcitabine chemotherapy in the current study. The PDGFR-β tyrosine kinase is thought to play a role in increased interstitial hypertension in tumors. In a syngeneic rat colon adenocarcinoma model, PDGFR-β expression was restricted to tumor stroma cells. After treatment with a DNA aptamer that inhibits PDGF-AB and PDGF-BB ligands, tumor interstitial pressure was significantly reduced (21). STI571 significantly reduced the interstitial pressure in KAT-4 anaplastic thyroid carcinoma tumors implanted s.c. in mice. The addition of STI571 also increased tumor uptake of [3 H]paclitaxel, increased the incidence of apoptosis, and decreased the rate of proliferation compared with either STI571 or paclitaxel alone. In both this model of thyroid carcinoma and a rat model of PROb colon cancer, the combination of STI571 with a chemotherapeutic agent produced a greater antitumor effect than either agent alone. The solid stress imparted by proliferating tumor cells may also increase vascular resistance and interstitial tumor pressure (22). Thus, decreasing the tumor proliferation rate could reduce interstitial...
pressure, thereby enhancing delivery of additional chemotherapeutic agents.

In addition to PDGF receptors located on tumor cells, endothelial cells may provide another target for inhibitors of PDGF activity. In this study, double immunofluorescence staining identified p-PDGF-R-β on endothelial cells in clinical specimens of human pancreatic adenocarcinoma as well as liver metastases from primary pancreatic adenocarcinomas. Treatment with STI571 inhibits phosphorylation of PDGF, which acts as a survival factor for endothelial cells (15). The addition of chemotherapy can therefore inhibit growth or induce apoptosis in both tumor cells and dividing tumor-associated endothelial cells. This can explain our finding that treatment with STI571 alone or in combination with gemcitabine decreased MVD in the mouse model of pancreatic cancer.

In conclusion, our results indicate that PDGF ligands and receptors are present in the majority (>90%) of human pancreatic adenocarcinomas. Inhibition of PDGF activity by STI571 in an orthotopic nude mouse model of pancreatic cancer decreased the growth of primary pancreatic tumors and decreased the incidence of peritoneal and liver metastases. The antitumor effect of STI571 was enhanced when it was combined with gemcitabine. The reduction in tumor volume was associated with increased apoptosis, decreased cell proliferation, and decreased MVD. The role of supporting elements such as endothelial cells and fibroblasts that surround pancreatic tumors should not be overlooked. The expression of PDGF and their receptors in these structures suggests that, in addition to the tumor cells, elements in stromal tissue associated with pancreatic cancer are important potential targets for PDGF antagonist therapy.

ACKNOWLEDGMENTS

We thank Kathryn Hale for critical editorial review and Lola López for expert assistance with the preparation of this manuscript.

REFERENCES


