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Simultaneous Inhibition of EGFR, VEGFR and PDGFR Signaling Combined with Gemcitabine Produces Therapy of Human Pancreatic Carcinoma and Prolongs Survival in an Orthotopic Nude Mouse Model

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Abstract

Although gemcitabine has been approved as the first-line chemotherapeutic reagent for pancreatic cancer, its response rate is low and average survival duration is still only marginal. Because epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR) modulate tumor progression, we hypothesized that inhibition of phosphorylation of all three on tumor cells, tumor-associated endothelial cells, and stroma cells would improve the treatment efficacy of gemcitabine in an orthotopic pancreatic tumor model in nude mice and prolong survival. We implanted L3.6pl, a human pancreatic cancer cell, in the pancreas of nude mice. We found that tumor-associated endothelial cells in this model highly expressed phosphorylated EGFR, VEGFR, and PDGFR. Oral administration of AEE788, a dual tyrosine kinase inhibitor against EGFR and VEGFR, decreased phosphorylation of EGFR and VEGFR. PDGFR phosphorylation was inhibited by STI571. Although intraperitoneal (i.p.) injection of gemcitabine did not inhibit tumor growth, its combination with AEE788 and STI571 produced >80% inhibition of tumor growth and prolonged survival in parallel with increases in number of tumor cells and tumor-associated endothelial cell apoptosis, decreased microvascular density, decreased proliferation rate, and prolonged survival. STI571 treatment also decreased pericyte coverage on tumor-associated endothelial cells. Thus, inhibiting phosphorylation of EGFR, VEGFR, and PDGFR in combination with gemcitabine enhanced the efficacy of gemcitabine, resulting in inhibition of experimental human pancreatic cancer growth and significant prolongation of survival.

Keywords

AEE 788; STI571; EGFR; VEGFR; PDGFR

INTRODUCTION

Pancreatic adenocarcinoma remains one of the most aggressive malignancies and is the fourth leading cause of cancer-related death in the United States (*1*). Because of difficulties in early diagnosis, only 10–20% of pancreatic cancers can be surgically resected with curative intent

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at the time of diagnosis (2). Most patients develop local recurrence and metastatic disease. Although gemcitabine can prolong survival of patients, only less than 3% survive 5 years after the initial diagnosis, and the median survival duration is less than 6 mo (3,4). Clearly, there is an urgent need to develop new treatment modalities for pancreatic cancer.

One general method under consideration is the modulation of cancer progression pathways and its interaction with the organ microenvironment. The epidermal growth factor (EGF) phosphorylates EGFR by binding to the EGFR and further stimulates multiple signaling pathways which are involved in cell proliferation (*e.g*., Ras/mitogen activated protein kinase [MAPK]), and anti-apoptosis (*e.g*., phosphatidylinositol 3-kinase(PI3k)/Akt, nuclear factor-kappa B [NF-κB]), and others (5–8). The overexpression of EGF and EGF-R by various type of malignancies has been shown to correlate with metastasis, apoptosis, resistance to chemotherapy and poor prognosis (9–11), indicating that inhibiting EGFR signaling is a good strategy for therapeutic intervention. Cetuximab (IMC C225, Erbitux, ImClone, New York, NY) is a monoclonal antibody to EGFR that inhibits binding of EGF to EGFR and stimulation of downstream signaling pathways (12). In locally advanced or pancreatic cancer expressing EGFR, Cetuximab in combination with gemcitabine produced a 12.2% partial response, and 63.4% of patients showed stable disease on a phase II clinical trial (13). Thus, inhibiting EGFR signaling in combination with gemcitabine for pancreatic cancer showed promising activity and has led to a phase III trial of Cetuximab plus gemcitabine.

Production of another growth modulator, vascular endothelial growth factor (VEGF), increased in most types of malignant tumors and is associated with angiogenesis and poor prognosis (14). VEGF is not only a proliferating and permeability factor but also an anti-apoptotic survival factor for vascular endothelial cells (15,16). Inhibiting VEGFR signaling could have a therapeutic efficacy not only by preventing angiogenesis, but also by causing vascular endothelial cells in the tumor microenvironment to regress. Bevacizumab (Avastin, Genentech, Inc., South San Francisco, CA) is a recombinant humanized monoclonal antibody to VEGF that inhibits its binding to VEGFR and activation of downstream signaling (17). In stage IV advanced pancreatic cancer patients, Bevacizumab in combination with gemcitabine produced a median survival of 9.0 months and a 74% 6-month survival. The partial response rate was 21%, and 45% of patients achieved stable disease, which are encouraging results (18). A randomized phase III trial of Bevacizumab plus gemcitabine is ongoing.

PDGF and its receptor are expressed in many types of cancer, including prostate, lung, gastric, and pancreatic (19,20). In our previous study, 29 of 31 human pancreatic cancer specimens expressed phosphorylated PDGFR (21). PDGFR signaling has been reported to increase proliferation of tumor cells in an autocrine manner (22,23) and to stimulate angiogenesis, recruit pericytes (which stabilize the tumor vasculature) (22,24), and control the interstitial fluid pressure in stroma to influence transvascular transport of chemotherapeutic agents in a paracrine manner (25,26). Inhibition of PDGFR activity by tyrosine kinase inhibitor STI571 (Novartis Pharma, Basel, Switzerland) (27) in an orthotopic nude mouse model of pancreatic cancer decreased the growth of primary pancreatic tumors and decreased the incidence of peritoneal metastases when combined with gemcitabine (21).

The most recent data indicate that the biological heterogeneity of neoplasms includes expression of tyrosine kinase receptors (22). Indeed, dual immunohistochemistry of human pancreatic cancer cells growing in the pancreas of nude mice revealed that tumor cells express both EGFR and PDGFR (Fig. 1) and, thus, inhibition of one receptor's signaling may not be sufficient to inhibit the progressive growth and spread of neoplasms. To overcome this heterogeneity and address the issue of redundancy in signaling pathways, we determined therapy of orthotopic human pancreatic cancer growing in nude mice by multiple PTK inhibitors. We examined whether the simultaneous inhibition of EGF-R, VEGF-R, and PDGF-

R signaling pathway in pancreatic tumor cells, tumor-associated endothelial cells, and stroma cells would increase the therapeutic efficacy of gemcitabine against pancreatic cancer. AEE788 (Novartis Pharma) is a novel synthesized small molecule inhibitor of both EGF-R and VEGF-R tyrosine kinases (29), and STI571 is an inhibitor of PDGF-R, Bcr-abl, and c-kit tyrosine kinase (27). We determined whether the oral administrations of AEE788 and/or STI571 administered alone or combined with intraperitoneal injections of gemcitabine inhibited the progressive growth of human pancreatic cancer cells implanted into the pancreas of nude mice and prolonged survival.

MATERIALS AND METHODS

Pancreatic Cancer Cell Line and Culture Condition

The human pancreatic cancer cell line L3.6pl was maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, a twofold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD) as described previously (21).

Nucleotide Sequence Analysis of EGFR in Pancreatic Cancer L3.6pl Cell Line

Mutations in exons 18, 19, and 21 of the kinase domain of EGFR have been shown to correlate with response of patients to therapy with the tyrosine kinase inhibitor Iressa (30). To exclude the possibility that the response to AEE788 was associated with mutation of the EGFR, we assayed DNA extracted from log phase cultures of L3.6pl cells using the DNeasy Tissue Kit No. 69504 (Qiagen, Inc., Valencia, CA). Mutational analysis was performed by the Molecular Diagnostic Laboratory of the M. D. Anderson Cancer Center (Houston, TX). Nested PCR products of exons 18, 19, and 21 obtained using primers previously described (30) were directly sequenced in sense and antisense directions. All sequences were screened for the presence of mutations both manually and using the SeqScape software and confirmed by two independent PCR amplifications. The results indicated that the L3.6pl cells contain a wild-type EGFR.

Reagents

AEE 788 (Novartis Pharma), 7H-pyrrolo[2,3-d]pyrimidine lead scaffold, is a low molecular weight, ATP-competitive dual EGF-R and VEGF-R tyrosine kinase family inhibitor (29). STI571 (imatinib mesylate or Gleevec; Novartis Pharma) is a 2-phenylaminopyrimidine class protein-tyrosine kinase inhibitor of PDGFR, BCR-ABL, and c-Kit (27). For oral administration, AEE 788 was diluted in DMSO and STI571 was diluted in sterile water. Gemcitabine (Gemzar, Eli Lilly Co, Indianapolis, IN) was maintained at room temperature and dissolved in phosphate buffered saline (PBS) on the day of use. It was administered by i.p. injection.

Primary antibodies were purchased from the following manufacturers: rabbit anti-pVEGFR 2/3 (Flk-1) (Oncogene, Boston, MA); rabbit anti-human, mouse, rat VEGF-R(Flk-1)(C1158) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-human pEGFR (Tyr 1173) (Biosource, Camarillo, CA); rabbit anti-human EGF and rabbit anti-human EGFR for paraffin samples (Santa Cruz Biotechnology); rabbit anti-human EGFR for frozen samples (Zymed, San Francisco, CA); rabbit anti-VEGF (A20) (Santa Cruz Biotechnology); polyclonal rabbit anti-PDGFR-β, polyclonal goat anti-phosphorylated PDGFR-β, and polyclonal rabbit anti-PDGF-β (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA); rat anti-mouse CD31 (BD PharMingen, San Diego, CA); mouse anti-proliferating cell nuclear antigen (PCNA) clone PC 10 (Dako A/S, Copenhagen, Denmark); and rabbit anti-desmin (Dako A/S)(as a pericyte marker). The following secondary antibodies were used for colorimetric immunohistochemistry: peroxidase-conjugated goat anti-rabbit IgG; F(ab')2 (Jackson

ImmunoResearch Laboratories, Inc., West Grove, PA); biotinylated goat anti-rabbit (Biocare Medical, Walnut Creek, CA); streptavidin horseradish peroxidase (Dako A/S); rat anti-mouse IgG2a horseradish peroxidase (Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN); and goat anti-rat horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.). The following fluorescent secondary antibodies were used: Alexa488 conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR) and Alexa 594 conjugated goat anti-rat IgG (Molecular Probes Inc.). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

Animals and Orthotopic Implantation of Tumor Cells

Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

To produce pancreatic tumors, L3.6pl cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in Hanks' balanced salt solution (HBSS). Only suspensions consisting of single cells with greater than 90% viability were used for injection into the pancreas of nude mice as described previously (21).

Treatment of Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice

Twenty-one days after the intra-pancreatic injection of 0.5×10^6 viable L3.6pl cells in 50 μl HBSS, the pancreatic tumors reached the size of 5–6-mm. At that time, the mice were randomized to the following 8 treatments (n=10): (1) Control mice: administration of water diluted at 1:20 with DMSO–0.5% Tween 80 (diluent) by oral gavage 3 times per week, daily oral gavage with sterile water, and i.p. injections of PBS twice a week; (2) administration of diluent by oral gavage 3 times per week, daily oral gavage with sterile water, and twice weekly i.p. injections of gemcitabine (50 mg/kg); (3) oral gavage of AEE788 (50 mg/kg), 3 times per week, daily oral gavage with sterile water, and twice per week i.p. injections of PBS; (4) oral gavage of AEE788 (50 mg/kg) three times per week, daily oral gavage with sterile water, and twice per week i.p. injection of gemcitabine (50 mg/kg); (5) daily oral gavage of STI571 (50 mg/kg), diluent of AEE788 by oral gavage 3 times per week and i.p. injections of PBS twice per week; (6) daily oral STI571 (50 mg/kg), oral gavage of diluent for AEE788 3 times per week, and i.p. injections of gemcitabine (50 mg/kg) twice weekly; (7) combination of oral AEE788 (50 mg/kg) 3 times per week, daily STI571 (50 mg/kg), and twice per week i.p. injections of PBS; and (8) combination of oral AEE788 (50 mg/kg) 3 times per week, STI571 (50 mg/kg) 7 times per week, and twice per week i.p. injections of gemcitabine (50 mg/kg). All mice were treated for 4 weeks and killed on day 49 of the experiment.

For survival studies, 21 days after the intra-pancreatic injection of 1.0×10^6 tumor cells in 50 μl HBSS, at which time the tumors in the pancreas exceeded 6- to 8-mm in diameter, the mice were randomized (n=10) to one of the 8 treatment groups, as described above. The mice were killed and necropsied when they became moribund. Survival was evaluated by the Kaplan-Meier method. The study was repeated.

Necropsy Procedures and Histological Studies

In the first treatment study, the mice were killed on day 49 after tumor cell injection, weighted, and necropsied. Tumors growing in the pancreas were excised and weighed. For immunohistochemical staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin and the other was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C.

Immunohistochemical (IHC) Analysis to Detect EGF, VEGF, PDGF-BB, EGFR, VEGFR, PDGFRβ pEGFR, pVEGFR, pPDGF-R in Pancreatic Tumors

Paraffin-embedded pancreatic tumors of mice from all treatment groups were immunostained to evaluate the expression of EGF, VEGF, PDGF-BB, EGFR, VEGFR, PDGFRβ, phosphorylated (p)EGFR, pVEGFR, and pPDGFRβ. The sections were deparaffinized in xylene, dehydrated with alcohol and rehydrated in PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. Samples were exposed to protein block (5% normal horse serum, 1% normal goat serum in PBS) and incubated overnight at 4°C with each primary antibody at the appropriate dilution. After 1 h incubation at room temperature with peroxidase-conjugated secondary antibody, positive reaction was detected by exposure to stable 3,3′-diaminobenzidine (DAB) (Phoenix Biotechnologies, Huntsville, AL). Slides were counterstained with Gill's #3 hematoxylin. Sections stained for immunoperoxidase or hematoxylin and eosin were examined in a Nikon Microphot-FX microscope equipped with a three-chip-charged coupled device (CCD) color video camera (Model DXC990, Sony Corp., Tokyo, Japan). Digital images were captured using Optimas Image Analysis software (Media Cybernetics, Silver Spring, MD).

IHC Determination of Proliferating Cell Nuclear Antigen (PCNA), CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptosis)

Paraffin-embedded tissues were used for IHC identification of proliferating cell nuclear antigen (PCNA). Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged slides, 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 (21). Control samples exposed to a secondary antibody alone showed no specific staining. For the quantification of mean vessel density (MVD) in sections stained for CD31, 10 random 0.159-mm^2 fields at X100 magnification were captured for each tumor, and microvessels were quantified. For quantification of PCNA expression, the number of positive cells was counted in 10 random 0.159-mm^2 fields at X100 magnification.

Analysis of apoptotic cells was performed by using a commercially available TUNEL kit (Promega) with the following modifications: Samples were fixed and incubated with an equilibration buffer followed by a reaction buffer (containing nucleotide mix and terminal deoxynucleotidyl transferase enzyme). Immunofluorescence microscopy was performed in a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an HBO 100 mercury lamp, narrow bandpass filters to individually select for green, red, and blue fluorescence (Chroma Technology Corp., Brattleboro, VT). Images were captured using a cooled CCD Hamamatsu Orca camera (Hamamatsu Corp., Bridgewater, NJ) and Image Pro Analysis software (Media Cybernetics, Silver Spring, MD). Photomontages were prepared using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA). The number of TUNEL-positive cells in 10 random 0.159-mm^2 fields at ×100 magnification was used to quantify apoptosis.

Double Immunofluorescence Staining for CD31/PECAM-1 and EGFR, pEGFR, VEGFR, pVEGFR, PDGFRβ, pPDGFRβ, Pericytes (desmin-positive cells), and TUNEL

Frozen sections of pancreatic tumors were mounted on slides and fixed. Immunofluorescence for CD31 was performed using Alexa594 conjugated secondary antibody, and samples were again blocked briefly in a blocking solution (5% normal horse serum and 1% normal goat serum in PBS) as described above and incubated with antibody against human EGFR, pEGFR, VEGFR, pVEGFR, PDGFRβ, pPDGFRβ, or desmin at 4°C overnight. After washes and blocking with blocking solution, samples were incubated with Alexa488 conjugated secondary antibody. Endothelial cells were identified by red fluorescence, and EGF-R, pEGFR, VEGFR, pVEGFR, PDGFRβ, pPDGFRβ and desmin positive cells (pericytes) were identified by green fluorescence. The presence of growth factor receptors and phosphorylated receptors on endothelial cells were detected by colocalization of red and green fluorescence, which appeared yellow.

The coverage of pericytes on endothelial cells was determined by counting CD31 positive cells in direct contact with desmin-positive cells and CD31-positive cells without direct association with desmin-positive cells in five randomly selected microscopic fields (at ×200 magnification) (31–33).

TUNEL-positive apoptotic cells were detected by localized green fluorescence within cell nuclei, and endothelial cells were identified by red fluorescence. Apoptotic endothelial cells were identified by yellow fluorescence within the nuclei. Quantification of apoptotic endothelial cells was expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in ten 0.159-mm^2 fields at ×100 magnification.

Statistical Analysis

Body weight, tumor weight, PCNA-positive cells, mean vessel density (CD31/PECAM-1), and TUNEL-positive cells were compared using the Mann-Whitney *U* test. Survival analysis was computed by the Kaplan-Meier method and compared by the Log rank test.

RESULTS

Therapy of Human Pancreatic Cancer Growing in the Cecum of Nude Mice

In the first set of experiments, the effect of treatment with AEE788, STI571, and gemcitabine alone and in various combinations was determined against well-established (5–6 mm) pancreatic tumors. The mice were killed and necropsied on day 49 of the study (Table1). Tumor incidence in the pancreas was 100% in all treatment groups. None of the treatments significantly affected body weight, indicating no obvious side effects. Control mice had the largest tumors (0.77 g). Treatment with STI571 or gemcitabine alone did not inhibit tumor growth, but mice treated with AEE788 had significantly smaller tumors (0.33g: $p<0.001$). The combination of AEE788 and gemcitabine or AEE788 and STI571 (but not STI571 and gemcitabine) significantly decreased tumor weight in the pancreas (0.19 g, $p<0.0001$, 0.33 g; $p<0.001$ vs control, and 0.71 g, respectively). Combining AEE788, STI571, and gemcitabine for therapy produced the most significant inhibition of tumor growth (0.14 g, $p<0.0001$ versus control).

In the next survival study, treatment began 21 days after the intrapancreatic injection of 1.0×10^6 L3.6pl cells. The pancreatic tumors measured 6–8 mm in diameter and thus were well established. Treatment continued until the mice became moribund, at which time they were killed. Survival was analyzed using the Kaplan-Meier method as shown in Figure 2. All treatments other than STI571 alone or gemcitabine alone significantly prolonged survival as

compared to the control treatment group. Mice treated with the combination of AEE788, STI571, and gemcitabine had the greatest prolongation of survival.

Immunohistochemical Analysis of L3.6pl Pancreatic Tumors

Tumor sections were analyzed immunohistochemically for the expression of EGF, EGFR, and pEGFR (Fig. 3A), VEGF, VEGFR, and pVEGFR (Fig. 3B), and PDGF-BB, PDGFRβ and pPDGFRβ (Fig. 3C). Treatment with AEE 788, STI571, gemcitabine, or any of the combination treatments did not alter the expression level of EGF, VEGF, PDGF-BB, EGFR, VEGFR, and PDGFRβ by the tumor cells or in the stroma cells. The phosphorylation of EGFR and VEGFR (but not PDGFR) was significantly reduced in tumors from mice treated with AEE788 alone or any combination therapy including AEE788 (Fig. 3A, 3B). In contrast, PDGFRβ (but not EGFR or VEGFR) phosphorylation was inhibited in tumors from mice treated with STI571 alone or combination therapy including STI571 (Fig. 3C). These data confirmed that at the concentration administered to mice, the PTK inhibitors produced specific inhibition of their respective target receptors. As expected, the combination therapies with AEE788 and STI571 and with AEE788, STI571, and gemcitabine inhibited phosphorylation of all three receptors.

EGF-R, VEGFR, PDGFR, pEGFR, pVEGFR and pPDGFR on Tumor-associated Endothelial Cells

To determine whether tumor-associated endothelial cells expressed EGFR, VEGFR, PDGFRβ, pEGFR, pVEGFR, or pPDGFRβ, we used a double immunofluorescence staining technique. Tumor-associated endothelial cells from all treatment groups expressed similar levels of EGFR (Fig. 4A), VEGFR (Fig. 4B), and PDGFRβ (Fig. 4C). The phosphorylation of EGFR and VEGFR was diminished on endothelial cells from tumors of mice treated with AEE788 or combination treatments including AEE788 (Fig. 4A, 4B).

Phosphorylation of the PDGFRβ was decreased on endothelial cells from tumors of mice treated with STI571 or combination treatments including STI571 (Fig. 4C). Administration of AEE788 and STI571 or AEE788, STI571, and gemcitabine inhibited phosphorylation of EGFR, VEGFR, and PDGFRβ on tumor-associated endothelial cells.

Cell Proliferation (PCNA), Apoptosis (TUNEL), and Mean Vessel Density (MVD)

Cell proliferation was evaluated by staining for PCNA (Fig. 5). In tumors from control mice, the median number of PCNA-positive cells was 371 ± 88. As shown in Table 2, treatment with gemcitabine alone or STI571 alone decreased the number of dividing PCNA-positive cells. A significant decrease of PCNA-positive cells was found in tumors from all other treatment groups, with the highest inhibition produced in tumors from mice treated with AEE788, STI571, and gemcitabine (155 ± 54, $P<0.001$).

The induction of apoptosis in the pancreatic tumors was evaluated by the TUNEL method (Table 2). In tumors from control-treated mice, the median number of apoptotic tumor cells was minimal (1 ± 1). The number of apoptotic cells in tumors from mice in all other treatment groups (except those treated with only STI571) increased, with the highest produced by therapy with the combination of AEE788, STI571, and gemcitabine (30 ± 10).

MVD in the tumors was determined by IHC staining with antibodies against CD31 (Table 2). The median number of CD31-positive tumor cells from control mice was 46 ± 11. Treatment with gemcitabine alone or STI571 alone did not decrease MVD. The number of CD31-positive cells was significantly decreased in tumors from all other treatment groups, with the largest decrease in MVD in tumors from mice treated with AEE 788, STI571, and gemcitabine (16 ± 6) ($P<0.001$).

Immunofluorescence Double Staining for CD31/PECAM-1 and TUNEL

Next, we determined whether therapy was associated with apoptosis of endothelial cells by using the CD31/TUNEL fluorescent double-labeling technique (Fig. 5B). Tumors from control mice had no apoptosis in tumor-associated endothelial cells. Treatment of mice with AEE788, STI571, and gemcitabine produced a median of 8 ± 5% apoptosis in tumor-associated endothelial cells (Table 2).

Pericyte coverage on tumor-associated endothelial cells

The effect of the different treatments on pericyte coverage on tumor-associated endothelial cells was evaluated using the double immunofluorescence staining technique with anti-CD31 antibody and anti-desmin antibody (Fig. 6A). Pericyte coverage rate in tumors from control-treated mice was 35.4 ± 9.8% (median ± S.D.). Treatment with STI571 alone or STI571 and gemcitabine produced a significant decrease in pericyte coverage ($p<0.05$, 18.8 ± 14.7%, 18.1 ± 10.3% respectively) (Fig. 6B). In contrast, treatment with gemcitabine alone, AEE788 alone, or treatment including AEE788 did not produce a measurable decrease in pericyte coverage. Thus, in this study, we did not find a correlation between inhibition of pericyte coverage of endothelial cells and a decrease in MVD.

DISCUSSION

The expression levels of EGF, VEGF, PDGF and their receptors have been reported to correlate with the progressive growth, metastasis, and resistance to chemotherapy of a variety of cancers (11,20,34,35). We previously reported that the majority (29/31) of human pancreatic cancer clinical specimens expressed PDGFR and pPDGFR (21). We also found that more than 80% of pancreatic cancer clinical specimens expressed EGF, VEGF, EGFR, VEGFR, pEGFR and pVEGFR on tumor cells and tumor-associated endothelial cells (Yokoi *et al.*, manuscript submitted). These data suggest that EGF-R, VEGF-R, and PDGF-R could be attractive targets for therapy of this cancer.

In the present study, human pancreatic cancer cells growing in the pancreas of nude mice expressed high levels of EGF, VEGF, PDGF-BB, and their receptors, and the receptors were phosphorylated. In addition to the tumor cells, tumor-associated endothelial cells also expressed these receptors, probably in response to specific ligands produced by tumor cells (19). Oral treatment with AEE788 inhibited the phosphorylation of EGFR and VEGFR (but not the expression of EGF, VEGF, EGFR and VEGFR) on pancreatic tumor cells and tumor-associated endothelial cells. Oral treatment with STI571 inhibited phosphorylation of PDGFR but did not alter PDGF-BB and PDGF-R expression levels. When AEE788 and STI571 were combined, phosphorylation of the EGFR, VEGFR, and PDGFR was inhibited on both the implanted human pancreatic cancer cells and the tumor-associated endothelial cells of the recipient mice.

L3.6pl cells growing in the pancreas of nude mice were resistant to treatment with gemcitabine (Fig. 1, Table 1). When combined with AEE788, however, gemcitabine reduced tumor growth by nearly 75% and significantly prolonged survival ($p<0.0001$). This therapeutic effect was significantly better than that from treatment with AEE788 alone ($p<0.05$). Indeed, the combination treatment using AEE788 and gemcitabine induced a significantly higher level of apoptosis in tumor and tumor-associated endothelial cells, decreased the number of proliferating cells, and a decreased MVD as compared to control. These data indicate that inhibition of both the EGFR and VEGFR signaling pathways on tumor cells and tumor-associated endothelial cells combined with a chemotherapeutic reagent is superior to either treatment administered alone.

STI571 as a single treatment had a limited effect on the inhibition of tumor growth and prolongation of survival. However, the combination of STI571 with AEE788 significantly lowered the number of PCNA-positive cells and the MVD and increased the number of apoptotic tumor cells and apoptotic endothelial cells; all these were associated with prolongation of survival. Similar data were produced by combining AEE788 with gemcitabine. The best therapy, however, was produced by combining AEE788 with STI571 and gemcitabine. This combination led to a decrease in tumor size, prolonged survival ($P<0.0001$), the fewest PCNA-positive tumor cells, the lowest MVD, and the highest number of apoptotic cells.

In our study, tumor-associated endothelial cells expressed not only EGFR and VEGFR, but also PDGFR, which would provide another target for inhibition of its signaling by STI571. PDGFR as well as EGFR and VEGFR signaling, which activates the anti-apoptotic protein Akt and bcl-2, acts like a survival factor for endothelial cells (36–38). With the inhibition of survival mechanisms by AEE788 and STI571, tumor-associated endothelial cells, whose proliferating frequency is 20–2000 times higher than that of endothelial cells in normal organs (39,40), would be more sensitive to anticycling chemotherapeutic treatment. Indeed, we found the largest number of apoptotic cells on tumor-associated endothelial cells (Table 2).

Until now, antiangiogenic therapy has focused mainly on endothelial cells. Recent studies, however, imply that pericyte can also play an important role in angiogenesis (22–24). Since pericyte recruitment and covering of endothelial cells for stabilization and maturation of vessel structure is dependent on PDGFRβ signaling (22), the inhibition of PDGFR signaling by a PTK inhibitor should inhibit pericyte recruitment and attachment to endothelial cells which would in turn confer resistance to VEGFR antagonists on endothelial cells (41,42). In agreement with other reports, we found that treatment with STI571 decreased pericyte coverage on tumor-associated endothelial cells, whereas AEE788 did not. However, administration of AEE788 seemed to reverse the effect of STI571, suggesting that AEE788 may target endothelial cells or targeted endothelial cells with relatively poor pericyte coverage.

The increased interstitial hyperpressure found in tumor stroma can decrease delivery of drugs. A number of studies reported that inhibition of PDGFR signaling can decrease this pressure and hence enhance the effects of chemotherapeutic reagents (25,26). Increased vascular permeability is a major reason for increased interstitial high pressure (43,44). Anti-VEGF mAb treatment can lower vascular permeability by normalization of vascular architecture and function (43). Taken together, these reports suggest that treatment with AEE788 and STI571 may decrease interstitial pressure as well as vascular permeability and, hence, increase delivery of gemcitabine to cancer cells.

In conclusion, pancreatic cancer cells produce EGF, VEGF, and PDGF. These ligands can activate their receptors on tumor cells by an autocrine manner and on tumor-associated endothelial cells by a paracrine manner. As a consequence, both tumor cells and tumor-associated endothelial cells have increased survival and resistance to chemotherapeutic agents (36). Inhibiting these signaling pathways by tyrosine kinase inhibitors combined with conventional chemotherapy induced a significant apoptosis in tumor-associated endothelial cells and tumor cells, resulting in decreased tumor size and significant prolongation of survival. The success of this multimodality therapy can be attributed to the heterogeneous nature of cancer. Targeting both tumor cells and tumor-associated endothelial cells can therefore be of great therapeutic benefit.

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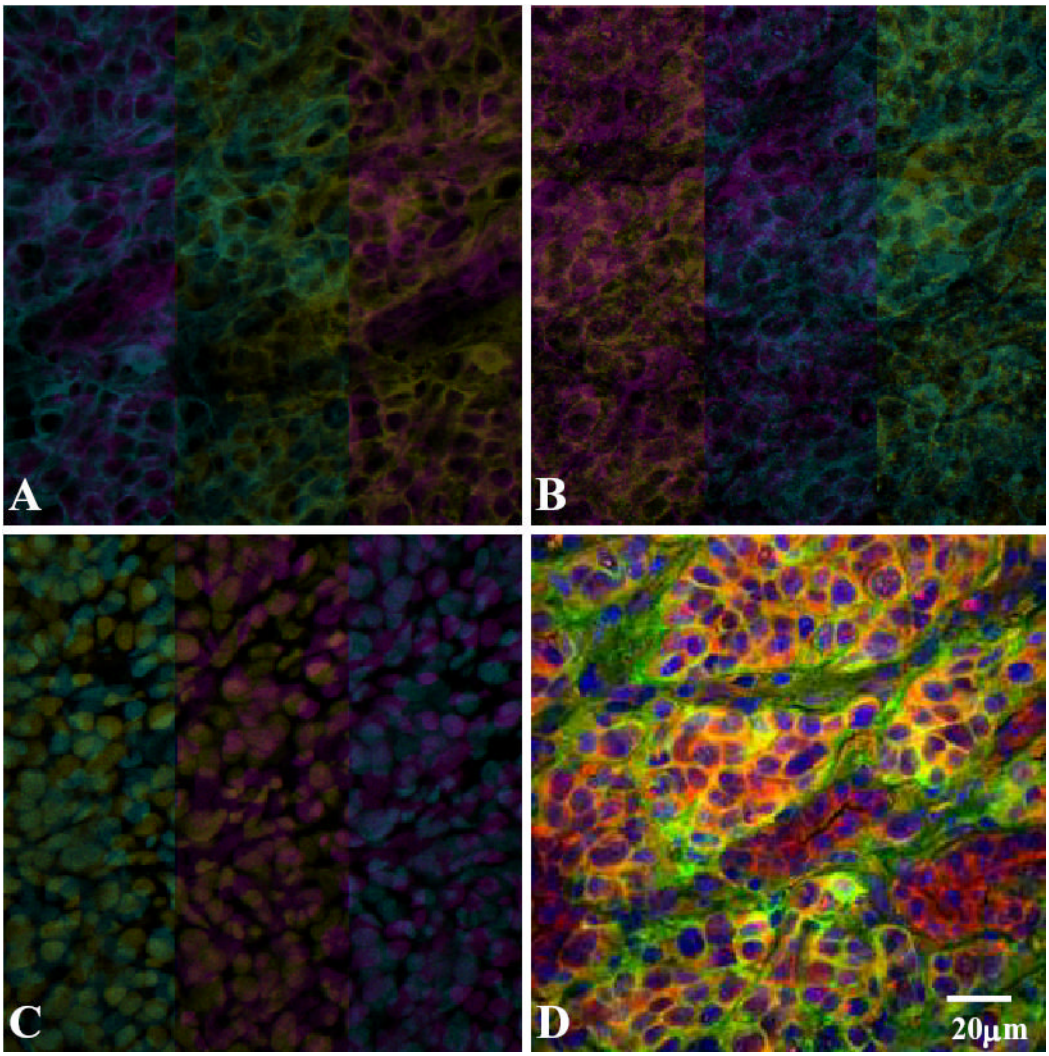


Fig. 1.

Double immunofluorescence staining for expression of EGFR and PDGFRβ in orthotopic L3.6pl tumor in nude mice. Samples were stained with anti-EGFR (A) and anti-PDGFRβ (B) antibodies as described in Materials and Methods. The nuclei were visualized by staining with Sytox green (C). Colocalization of EGFR and PDGFRβ appears as yellow fluorescence (D).

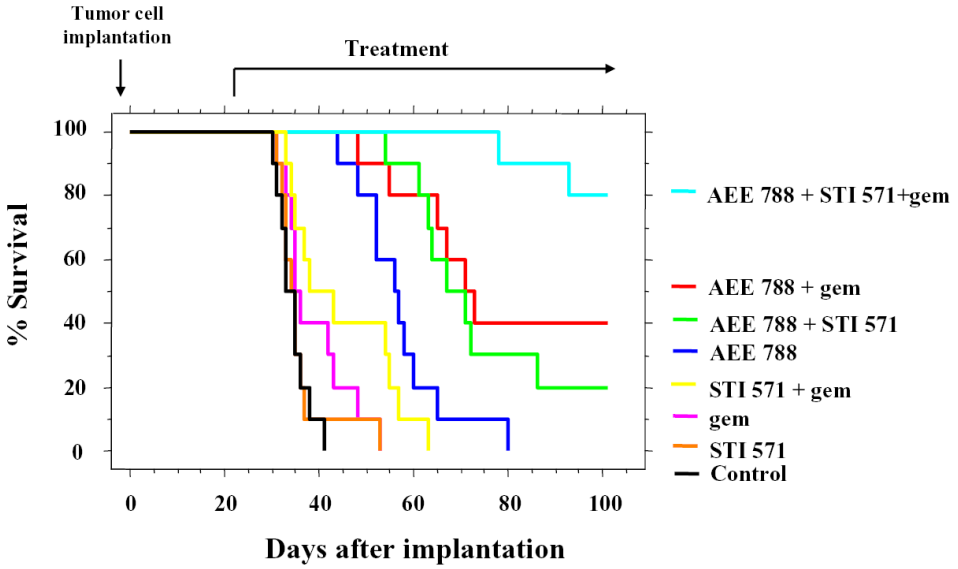


Fig. 2.

Therapeutic effects of AEE788, STI571, gemcitabine and their combinations on survival rate. Nude mice were injected with L3.6pl human pancreatic cancer cells (1×10^6) into the pancreas. Twenty-one days after the injection, the mice were randomized into 8 treatment groups (n=10) as detailed in Table 1. Mice were killed when got moribund. Survival analysis was done by the Kaplan-Meier method and compared by the Logrank test. AEE 788 + STI571 + Gemcitabine: $p<0.0001$ vs Control, STI, Gem, STI+Gem, $p<0.001$ vs AEE, $p<0.01$ vs AEE +STI, $p<0.05$ vs AEE+Gem. AEE788+Gemcitabine: $p<0.0001$ vs Control, STI, Gem, STI +Gem, $p<0.05$ vs AEE. AEE788+STI571: $p<0.0001$ vs Control, STI, Gem, STI+Gem, $p<0.001$ vs AEE. AEE788: $P<0.0001$ vs Control, $p<0.01$ vs STI, STI571+Gemcitabine: $p<0.05$ vs Control

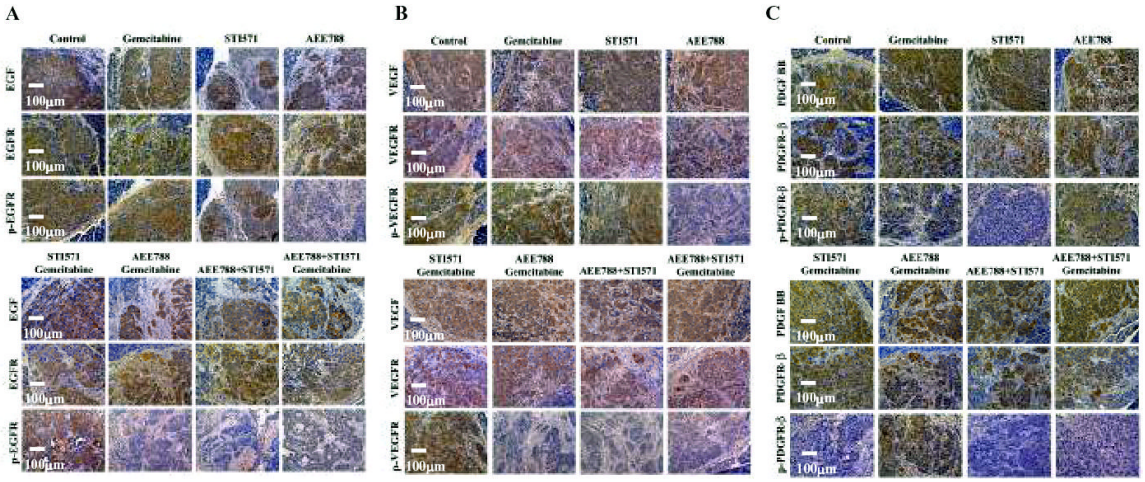
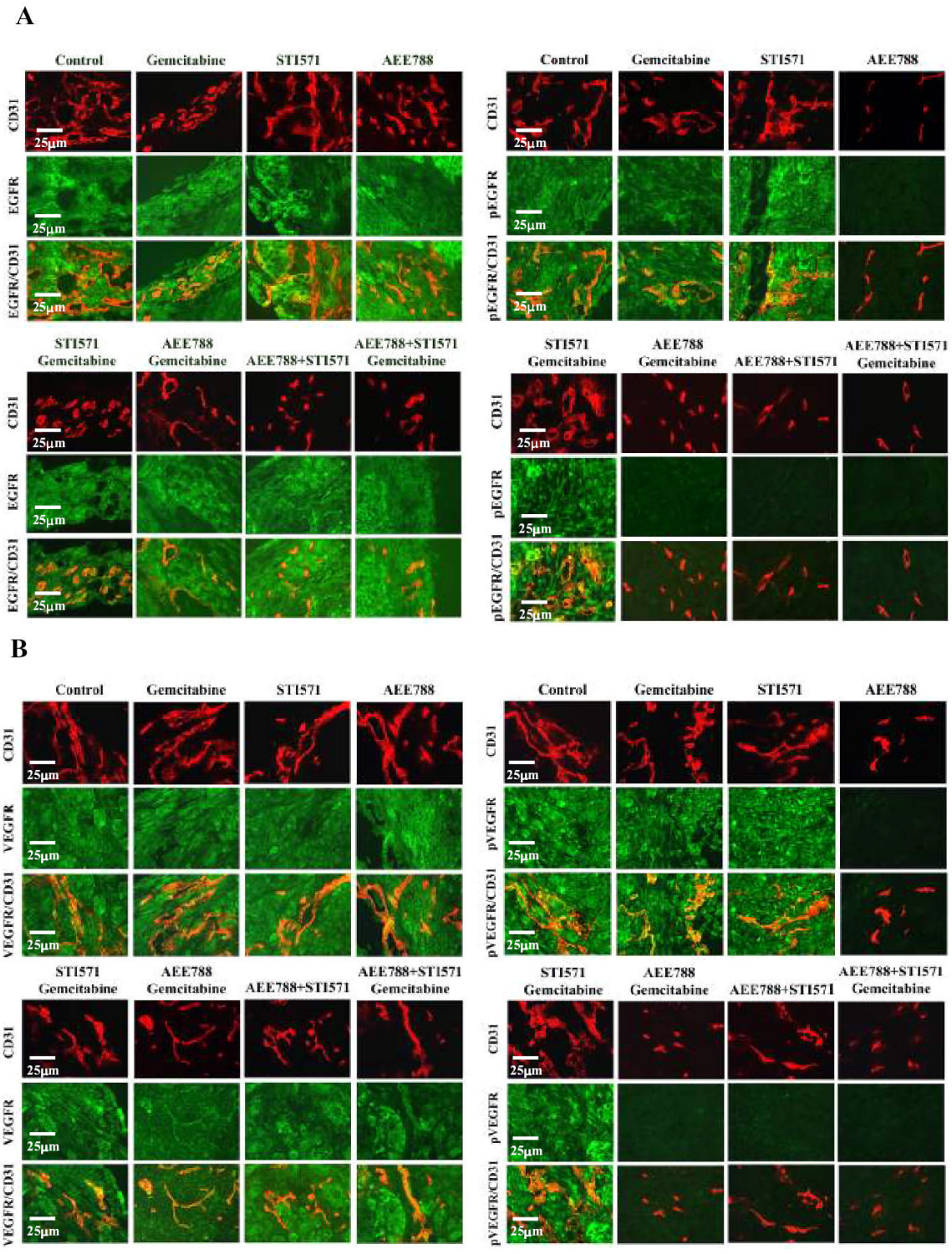


Fig. 3.

Immunohistochemical analysis of the expression of EGF, EGFR, pEGFR, VEGF, VEGFR, pVEGR and PDGF-BB, PDGFRβ and pPDGFRβ. L3.6pl human pancreatic cancer cells growing in the pancreas of nude mice were treated as described in Materials and Methods for 4 weeks, and all the mice were killed on day 28. Tumor tissue sections were stained for EGF, EGF-R, p-EGF-R (A), VEGF, VEGF-R, p-VEGR (B) and PDGF-BB, PDGFRβ, pPDGFRβ (C) as described in Material and Methods. Tumors from all treatment groups expressed similar levels of the ligands and receptors. Tumor from mice treated with AEE788 showed decreased phosphorylation of EGFR and VEGFR and tumor from mice treated with STI571 showed inhibition of phosphorylation of PDGFRβ. Combination of AEE788 and STI571 inhibited phosphorylation of EGFR, VEGFR, and PDGFRβ.



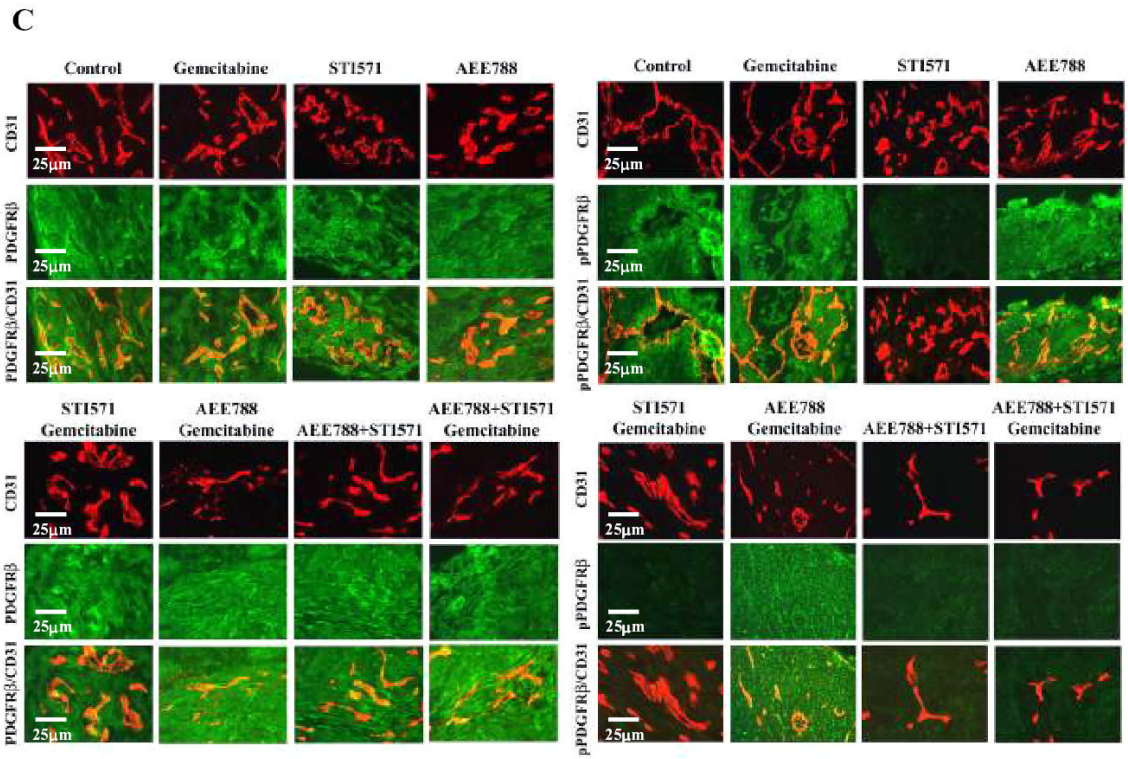


Fig. 4.

Double immunofluorescence staining for CD31/PECAM-1 and EGFR, pEGFR VEGFR, pVEGFR, PDGFRβ or pPDGFRβ in pancreatic tumors. Tumor sections were stained with anti-CD31/PECAM1 antibody (red) and anti-EGFR, pEGFR(A), VEGFR, pVEGR(B), PDGFRβ or pPDGFRβ (C) in green fluorescence as described in Materials and Methods. Colocalization of CD31 and EGF-R, pEGFR, VEGFR, pVEGR, PDGFRβ, or pPDGFRβ appears in yellow fluorescence. Expression of EGFR, VEGFR or PDGFRβ by tumor-associated endothelial cells was found in tumors from all treatment groups. Phosphorylation of EGF-R and VEGFR on endothelial cells was decreased by treatment with AEE 788 and phosphorylation of PDGFRβ on tumor-associated endothelial cells was decreased by treatment with STI571. Combination of AEE788 and STI571 inhibited phosphorylation of EGFR, VEGFR and PDGFRβ simultaneously.

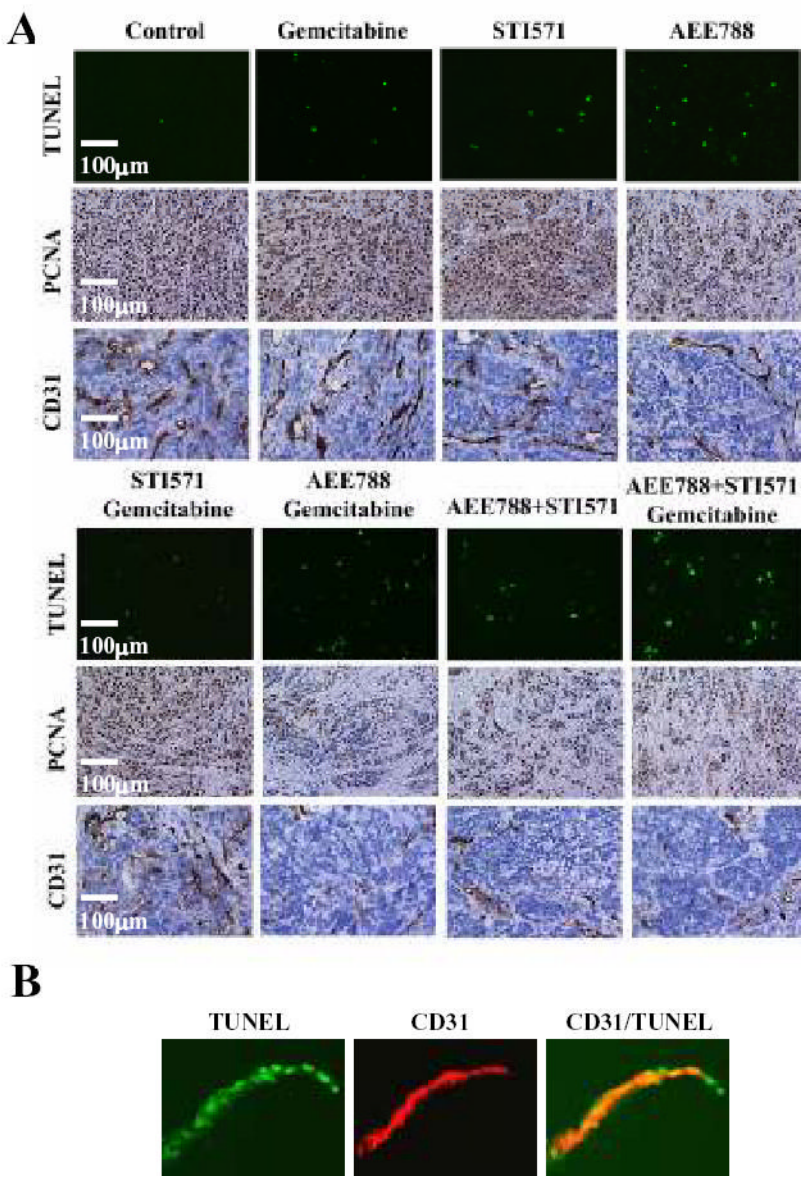


Fig 5.

(A) Analysis of apoptosis (TUNEL), cell proliferation (PCNA), and microvessel density (CD31). Mice were treated with control, gemcitabine, AEE 788, STI571, or the combination of AEE 788 and gemcitabine, STI571 and gemcitabine, AEE788 and STI571, or AEE788 and STI571 and gemcitabine. Pancreatic tumors were resected and processed for immunohistochemical evaluation of PCNA, TUNEL, and CD31 as described in Materials and Methods. (*B*) Double immunofluorescence staining of CD31/PECAM-1 and TUNEL in pancreatic tumors from mice treated with the combination of AEE788, STI571, and gemcitabine. Endothelial cells (CD31+) stained red fluorescence and apoptotic cells ($TUNEL^+$) stained green fluorescence. Colocalization of endothelial cells undergoing apoptosis yielded yellow fluorescence.

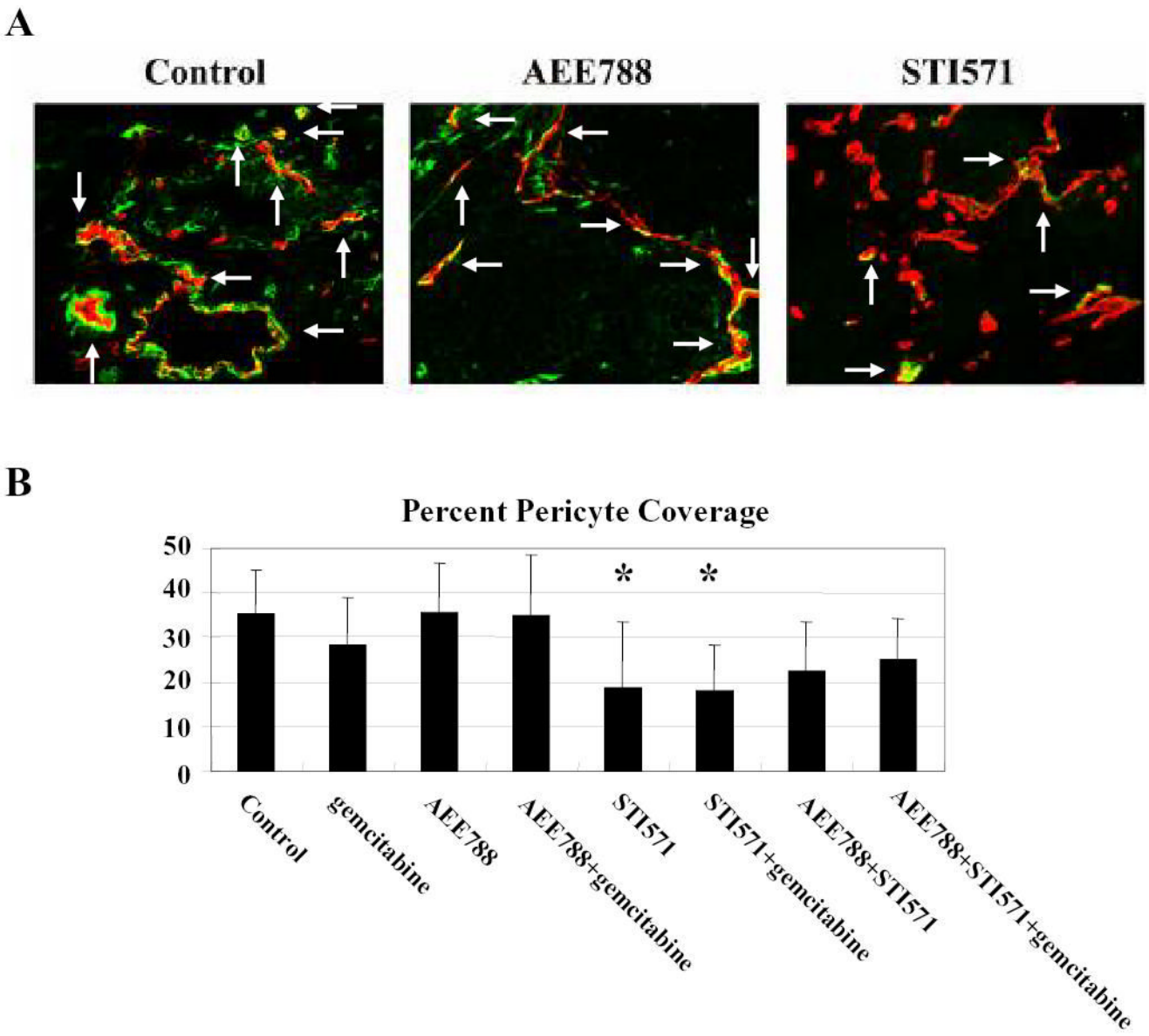


Fig 6.

Pericyte coverage on tumor-associated endothelial cells in the pancreatic tumors. Tumor sections were stained with anti-CD31/PECAM1 antibody (red) and anti-desmin antibody (pericyte marker) in green fluorescence, and the pericyte coverage rate was determined as described in Materials and Methods. Representative photomicrographs of pericyte coverage from control, AEE788, and STI571 treatment groups. Arrowhead indicates pericyte coverage of tumor-associated endothelial cells (A). Pericyte coverage rate was significantly decreased by STI571 or combination with STI571 and gemcitabine treatment as compared to those in control (B). *: $p<0.05$ versus control.

Table 1

Therapy of L3.6pl human pancreatic cancer cells implanted in the pancreas of nude mice

Treatment	Body weight(g)		Tumor weight (g)	
	Median	(Range)	Median	(Range)
Control	24.8	(18.8–27.8)	0.77	(0.48–1.80)
Gemcitabine	25.7	(20.0–28.1)	0.78	(0.36–1.23)
STI571	23.5	(18.7–27.2)	0.96	(0.45–1.83)
STI571 + Gemcitabine	25.0	(21.1–28.1)	0.71	(0.42–1.35)
AEE788	26.2	(21.3–28.5)	0.33	(0.08–0.44)[a]
AEE788 + Gemcitabine	25.3	(22.1–28.8)	0.19	(0.05–0.40)[b]
AEE788 + STI571	24.1	(22.2–29.0)	0.33	(0.05–0.50)[a]
AEE788 + STI571 + Gemcitabine	24.0	(21.5–28.9)	0.14	(0.04–0.30),[bc]

L3.6pl cells (0.5×10^6) were injected into the pancreas of nude mice. Three weeks later, the mice were randomized (n=10) to receive the following regimens: (1) Control: oral and i.p. diluent only; (2) Gemcitabine: twice per week i.p. injection of gemcitabine (50 mg/kg); (3) STI571: daily oral gavage of STI571 (50 mg/kg); (4) STI571 and Gemcitabine: combination of oral STI571 (50 mg/kg) and i.p. injection of gemcitabine (50 mg/kg) twice weekly; (5) AEE788: oral gavage of AEE788 (50 mg/kg) 3 times per week; (6) AEE788 and Gemcitabine: Combination of oral AEE788 (50 mg/kg) and twice per week i.p. injection of gemcitabine (50 mg/kg); (7) AEE788 and STI571: Combination of oral AEE788 (50 mg/kg) 3 times per week and STI571 (50 mg/kg) daily; (8) AEE788, STI571, and Gemcitabine: Combination of oral AEE788 (50 mg/kg) 3 times per week, STI571 (50 mg/kg) daily, and i.p. injection of gemcitabine (50 mg/kg) twice weekly. All mice were treated for 4 wk and killed on day 49 of the study. Body weight, tumor incidence, and tumor weight were recorded. All mice had pancreatic tumors.

[a] $P<0.001$ vs control.

[b] $P<0.0001$ vs control.

[c] $P<0.05$ vs AEE788 or AEE788 and STI571.

Table 2

Immunohistochemical analysis of L3.6pl human pancreatic cancer cells growing in the pancreas of nude mice

Treatment	Tumor cells		Endothelial cells	
	PCNA,[ac]	TUNEL,[bc]	CD31[c]	TUNEL$^+$ (%)[c]
Control	371 ± 88	1 ± 1	46 ± 11	0 ± 0
Gemcitabine	305 ± 71	8 ± 3[f]	38 ± 7	1 ± 1
STI571	301 ± 49	6 ± 2	37 ± 7	0 ± 0
STI571 + Gemcitabine	254 ± 48[e]	11 ± 4[f]	34 ± 8[d]	0 ± 1
AEE788	233 ± 54[e]	14 ± 4[f]	25 ± 5[d]	3 ± 3[e]
AEE788 + Gemcitabine	187 ± 48[f]	22 ± 7,[fk]	28 ± 7,[fk]	8 ± 6[e]
AEE788 + STI571	204 ± 69[e]	18 ± 6[f]	21 ± 5[f]	5 ± 5[e]
AEE788 + STI571 + Gemcitabine	155 ± 54,[fh]	30 ± 10,[gij]	16 ± 6,[fI]	8 ± 5[e]

[a] PCNA, proliferating cellular nuclear antigen;

[b] TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling;

[c] Median ± S.D.;

[d] $P<0.05$ vs control;

[e] $P<0.01$ vs control;

[f] $P<0.001$ vs control;

[g] $P<0.001$ vs control;

[h] $P<0.05$ vs AEE788;

[i] $P<0.01$ vs AEE788;

[j] $P<0.05$ vs AEE788 + STI571;

[k] $P<0.05$ vs AEE788.