Effect of Rapamycin Alone and in Combination with Antiangiogenesis Therapy in an Orthotopic Model of Human Pancreatic Cancer

Susann Stephan,1 Kaustubh Datta,1,5,6 Enfeng Wang,1,5,6 Jinping Li,1,5,6 Rolf A. Brekken,3 Sareh Parangi,2 Philip E. Thorpe,4 and Debabrata Mukhopadhyay1,5,6

Departments of Pathology and Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; Departments of Surgery and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas; and 3Mayo Clinic Cancer Center and 4Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota

ABSTRACT

Purpose: The overall 5-year survival of patients with pancreatic cancer remains <5%. Novel therapeutic strategies are needed. We examined the effect of rapamycin, alone and in combination with antiangiogenesis therapy, on pancreatic cancer in vivo.

Experimental Design: Human pancreatic cancer AsPC-1 cells were orthotopically injected into severe combined immunodeficient/beige mice to evaluate primary tumor growth and liver metastasis after treatment with rapamycin alone or in combination with anti-vascular endothelial growth factor antibody 2C3. Tumor cell proliferation was determined by bromodeoxyuridine incorporation. To detect tumor cell apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used. Tumor angiogenesis was investigated by using a monoclonal anti-CD31 antibody. All statistical tests were two-sided.

Results: Rapamycin, alone and in combination with 2C3, strongly inhibited primary and metastatic tumor growth in an orthotopic pancreatic cancer animal model. Furthermore, the combination therapy significantly improved the effect on liver metastasis compared with single treatment with either rapamycin (P = 0.0128) or 2C3 (P = 0.0099). Rapamycin alone inhibited pancreatic tumor cell proliferation, induced apoptosis, and decreased tumor angiogenesis. Nevertheless, the combination therapy showed a significant, stronger inhibition of tumor cell proliferation (P = 0.0002 versus rapamycin alone and P < 0.0001 versus 2C3 alone). The induction of apoptosis was significantly higher than in the rapamycin-treated group (P = 0.0039). Additionally, the combination therapy further improved suppression of tumor cell angiogenesis compared with rapamycin treatment (P = 0.029).

Conclusions: Our studies propose new therapeutic strategies to inhibit both primary and metastatic tumor growth in pancreatic cancer. Considering the fact that liver metastasis is a crucial problem in advanced stages of pancreatic cancer, the combination therapy of rapamycin plus anti-vascular endothelial growth factor antibody 2C3 is a significant advantage compared with single treatment with rapamycin.

INTRODUCTION

Adenocarcinomas of the pancreas are the fourth-leading cause of death in North America among men and women of all ages and the third cause of death among men between the ages of 40 and 59 years. More than 50% of the patients have advanced-stage disease at diagnosis. The 5-year survival rate among those patients remains about 2% (1). Although there have been encouraging results with the use of gemcitabine as a standard first-line agent for the treatment of advanced pancreatic cancer, a recent phase III trial has shown an overall median survival of 5.4 months for treatment with gemcitabine alone. A combination therapy of gemcitabine plus 5-fluorouracil did not improve the attained results (2). Therefore, novel therapeutic strategies are required to improve the prognosis of patients with pancreatic cancer. Among these strategies are drugs that target signal transduction pathways involved in tumor cell proliferation, invasion, or tumor angiogenesis.

One of the most promising drugs may be rapamycin, a bacterial macrolide with antifungal, immunosuppressant, and antitumor activities. Rapamycin is known to target the atypical Ser/Thr kinase mammalian target of rapamycin (mTOR) and inhibits the translation of key mRNAs of proteins required for cell cycle progression. Rapamycin binds with high affinity to the cytosolic 12-kDa FK506-binding protein (FKBP12). The rapamycin/FKBP12 complex inhibits interleukin-2–stimulated signal transduction and causes a partial dephosphorylation and deactivation of p70S6 kinase, an enzyme critical for the G1 to S transition (3). Furthermore, rapamycin inhibits mitogen-stimulated phosphorylation of 4E-BP-1 (4). Dephosphorylated 4E-BP-1 interacts with the translation initiation factor eIF-4E and thereby inhibits cap structure-dependent protein synthesis.
and cell growth (5). Due to the inhibitory effect of rapamycin on interleukin-2–stimulated T-cell division, rapamycin is approved as an immunosuppressive drug for prevention of allograft rejection after renal transplantation. Interestingly, recent experiments with immunosuppressant doses of rapamycin indicated an inhibition after renal transplantation. Interestingly, recent experiments as an immunosuppressive drug for prevention of allograft rejection.

Another strategy involves targeting angiogenic factors that are important for the growth of new blood vessels from a preexisting microvascular bed. The blood supply delivers oxygen and nutrients to the tumor cells and contributes to metastatic spread. Vascular permeability factor/vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors described thus far. An increased expression of vascular permeability factor/VEGF in adenopancreatic carcinoma cells is associated with liver metastasis and a poor prognosis for the patient (7). Thus, antiangiogenic drugs may represent a promising therapeutic option.

Because tumorigenesis has been shown to be a multifaceted process involving a variety of potential therapeutic targets, further improvement may be achieved by combining different therapeutic concepts. In this study, we describe experiments indicating that immunosuppressive doses of rapamycin inhibit primary and metastatic tumor growth in pancreatic cancer in vivo. Furthermore, we show that a combination therapy of rapamycin, administered in conjunction with an angiogenesis inhibitor, improves the results achieved in the single-treatment groups.

**MATERIALS AND METHODS**

**Drugs.** Rapamycin isolated from *Streptomyces hygroscopicus* was obtained from Sigma-Aldrich (St. Louis, MO). A carrier solution was produced by using a diluent containing Tween 80 (10%), N-N-dimethylacetamide (20%), and polyethylene glycol 400 (70%; all from Sigma-Aldrich).

The VEGF antibody 2C3 is a mouse IgG2a monoclonal antibody developed to target recombinant human VEGF as described previously (8). 2C3 prevents VEGF from binding to VEGF receptor (VEGFR) 2 (KDR/Flik-1), but not VEGFR1 (Flt-1), in enzyme-linked immunosorbent and coprecipitation assays performed in solution. 2C3 is specific to human VEGF (both VEGF121 and VEGF165).

**Human Pancreatic Adenocarcinoma Cell Line.** The human pancreatic adenocarcinoma cell line AsPC-1 was purchased from American Type Culture Collection (Manassas, VA). AsPC-1 cells were cultured in RPMI 1640 with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

**Orthotopic Tumor Model.** Female 6-week–old severe combined immunodeficient (SCID)/beige mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in the institutional animal facilities. All animal work was performed under protocols approved by the Beth Israel Deaconess Medical Care Institutional Animal Care and Use Committee. To establish tumor growth in mice, animals were anesthetized by subcutaneously administering Avertin [2,2,2 tribromethanol; Sigma-Aldrich], 2-methyl-2-butanol (Fisher-Scientific, Pittsburgh, PA), and tert-amyl alcohol (Fisher Scientific) at doses of 0.2 mL per 10 g of body weight. Then 2 × 10⁶ AsPC-1 cells, resuspended in 200 μL of RPMI 1640, were injected directly into the pancreas. Tumors were allowed to grow for 3 days without treatment. On day 4 after tumor cell injection, mice were randomized into four groups (eight animals per group), and treatment was initiated. One group was treated with rapamycin alone, administered at doses of 1.5 mg/kg/d intraperitoneally. A second group was treated with anti-VEGF antibody 2C3 alone, given at 100 μg per injection intraperitoneally three times per week during the first week and twice during the second and third week. In a third group, both rapamycin and anti-VEGF antibody 2C3 were administered by using the same schedule for each drug as described for the single treatments. The control group received daily injections of the carrier solution intraperitoneally. All mice were sacrificed by asphyxiation with CO₂ on day 20, and tumors were removed, measured, and prepared for immunohistochemistry. Primary tumor volumes were calculated by the formula \( V = \frac{1}{2}a \times b^2 \), where \( a \) is the longest tumor axis, and \( b \) is the shortest tumor axis.

**Cell Proliferation Assay.** To perform the tumor cell proliferation assay in vivo, we used a technique that incorporates bromodeoxyuridine (BrdUrd) into proliferating cells (S-phase). All animals received intraperitoneal injection with 1 mL of BrdUrd labeling reagent (Zymed Laboratories, South San Francisco, CA) per 100 g of body weight 2 hours before sacrifice. Then tumor samples were removed, fixed in neutral buffered 10% formalin overnight, and embedded in paraffin. Tissue sections were cut and mounted on slides. After slides were pretreated by heating at 60°C, tissue samples were deparaffinized and rehydrated by washing in xylene and a graded series of ethanol. Endogenous peroxidase was blocked with 30% hydrogen peroxide. Slides were then rinsed with PBS and digested with trypsin (Zymed Laboratories). Nonspecific binding was blocked (blocking solution; Zymed Laboratories). BrdUrd was stained with a biotinylated mouse monoclonal anti-BrdUrd antibody (Zymed Laboratories). Slides were then incubated with streptavidin peroxidase, and peroxidase activity was visualized with 3,3′-diaminobenzidine (Zymed Laboratories). Counterstaining was done with hematoxylin. The stained sections of five tumors from each group were reviewed, and BrdUrd-positive nuclei were scored in 10 randomly selected high-power fields (magnification, ×40).

**Detection and Quantitation of Apoptosis.** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Intergen Co., Purchase, NY) was used for proving apoptosis in individual cells. The procedure was carried out following the manufacturer’s directions using an ApopTaq Plus peroxidase in situ detection kit (Intergen). Briefly, after deparaffinization and rehydration in xylene and a graded series of ethanol, tissue sections were incubated with proteinase K (20 μg/mL) for 15 minutes at room temperature. Endogenous peroxidase was inhibited with 3% hydrogen peroxide for 5 minutes at room temperature. Terminal deoxynucleotidyl transferase was then applied to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of the fragmented DNA for 1 hour at 37°C. Subsequently, the slides were incubated with a horseradish peroxidase-conjugated antidigoxigenin antibody. To visualize peroxidase activity, 3′,3′-diaminobenzidine substrate was added. Sections were counterstained with methyl green. Sections from normal female rat mammary gland (pro-
vided by Intergen), in which extensive apoptosis occurs, served as a positive control. Negative controls were run in which terminal deoxynucleotidyl transferase was omitted. The stained sections of three tumors of each group were reviewed, and TUNEL-positive cells were scored in 10 randomly selected high-power fields (magnification, ×40).

To detect the human Fas receptor (CD95), we used formalin-fixed, paraffin-embedded tumor tissue. The Fas mouse monoclonal antibody was obtained from NovoCastra (Newcastle upon Tyne, United Kingdom). Cells were counted in 10 randomly selected high-power fields (magnification, ×40) in 3 of 8 animals per group, and the average was calculated.

Quantitation of Blood Vessels in Solid Tumors. We determined the total number of blood vessels in cross-sections of three solid pancreatic tumors of each group with a rat monoclonal antibody to mouse CD31 (PharMingen, San Diego, CA). Briefly, tumor tissues were immersed in freshly prepared 4% paraformaldehyde in 0.02 mol/L phosphate buffer (pH 7.4). After 4 hours at room temperature, tissues were transferred to 30% sucrose in PBS (pH 7.4) and incubated overnight at 4°C before embedding in OCT compound (Ted Pella, Inc., CA). Frozen 5-μm to 10-μm cryostat sections were collected on slides, fixed in ice-cold acetone and 80% methanol, and rehydrated in PBS. Non-specific binding was blocked in 20% fetal calf serum and 10% normal goat serum for 20 minutes. Primary antibody was added, and slides were incubated for 60 minutes at room temperature. Endogeneous peroxide was blocked with 30% hydrogen peroxide. Secondary antibody (biotinylated anti-rat IgG; Vector Laboratories, United Kingdom) was added at 1:200. ABC Elite kit solution (Petersborough; Vector Laboratories) was added at 1:200, and slides were incubated for 30 minutes at 4°C. Slides were then placed in staining solution [3-amino-9-ethyl-carbazole in N,N-dimethyl formamide, 0.1 mol/L acetate buffer (pH 5), and 30% hydrogen peroxide] for 5 minutes. To stop the staining reaction, slides were placed in 4% formaldehyde-acetate buffer (pH 5) for 10 minutes. Counterstaining was done with hematoxylin.

Liver Metastasis. Intrahepatic tumor growth was evaluated in tissue sections from six animals of the control group, five animals of the combination therapy-treated group, and four animals from the single-treatment groups. Briefly, the entire liver of each animal was fixed in neutral-buffered 10% formalin overnight and embedded in paraffin. Tissue sections were then mounted on slides and processed for staining with hematoxylin and eosin following standard protocols. Histologic views were digitalized, metastases were outlined, and the hepatic replacement area was calculated from the ratio of tumor area to total liver area (IP Lab software).

Statistical Analysis. Tumor volumes were summarized by means and 95% confidence intervals (CIs). BrdUrd-labeled nuclei, CD31-stained blood vessels, and TUNEL-positive cells were counted as described above, and means and 95% CIs were calculated. The hepatic replacement area was summarized as described above; data are given as mean and 95% CIs. To determine the statistical difference among the groups, Gaussian distribution was tested by using the method of Kolmogorov and Smirnov. Groups that followed the Gaussian distribution were tested by parametric tests (analysis of variance for differences among all four groups; unpaired Student’s t test for differences among two groups). Groups that did not follow Gaussian distribution were log-transformed or tested by nonparametric tests [Kruskal-Wallis test (nonparametric analysis of variance) for differences among all four groups; Mann-Whitney test for differences among two groups]. Two-tailed P values of <0.05 were considered statistically significant.

RESULTS

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Primary Tumor Growth in Pancreatic Cancer In vivo. To investigate the effect of rapamycin on tumor growth in pancreatic cancer in vivo, we injected AsPC-1 human pancreatic adenocarcinoma cells into the pancreas of SCID/beige mice, simulating orthotopic primary tumor growth. Rapamycin was administered at doses of 1.5 mg/kg/d intraperitoneally, beginning on day 4 after tumor cell implantation. All control mice received an equal volume of carrier solution intraperitoneally. By day 20 relative to tumor cell injection, we found a significant suppression in primary tumor growth among mice treated with rapamycin alone in comparison to the control group. The average tumor size was 0.36 cm³ (95% CI, 0.25–0.46) in the control group and 0.074 cm³ (95% CI, 0.042–0.11) in the rapamycin-treated group (P = 0.0002, Mann-Whitney test versus control group; Fig. 1A and B).

To evaluate whether the effect of rapamycin on pancreatic primary tumor growth can be improved by a combination of rapamycin with anti-angiogenesis therapy, we used a mouse IgG2a monoclonal VEGF antibody (2C3) that prevents VEGF from binding to its receptor, VEGF-R2/KDR. The treatment was initiated on day 4 after tumor cell injection. To monitor the effect of anti-VEGF antibody alone, 2C3 was administered as a single agent intraperitoneally three times within the first week and twice within the second and third week. The combination therapy of rapamycin + 2C3 was administered by using the same schedule for each drug as described for the single treatment. On day 20 relative to tumor cell injection, mice treated with 2C3 showed a significant suppression of primary tumor growth. The observed average tumor size was 0.16 cm³ (95% CI, 0.13–0.19; P = 0.0002, Mann-Whitney test versus control group). The highest suppression of primary tumor growth was found in mice treated with the combination therapy. The average tumor size was 0.057 cm³ (95% CI, 0.033–0.081; P = 0.0002, Mann-Whitney test versus control group). The combination therapy significantly improved the results achieved in the 2C3-treated group (P = 0.0002, Mann-Whitney test for comparison between combination therapy-treated group and 2C3-treated group). Although animals receiving the combination therapy showed an 18.6% smaller tumor volume than animals treated with rapamycin alone, this difference did not reach statistical significance (P = 0.3282, Mann-Whitney test for comparison between combination therapy-treated group and rapamycin-treated group, Fig. 1A and B).
Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Metastatic Tumor Growth in Pancreatic Cancer In vivo.

The selected orthotopic tumor model also mimics the metastatic progression of pancreatic cancer as observed in human pancreatic tumor patients. Histologic analysis of the entire liver tissue showed an average hepatic tumor replacement area of 0.47% (95% CI, 0.24–0.70) measured in the control group 20 days after tumor cell injection. The animals treated with single-agent rapamycin therapy showed a significant suppression of metastatic tumor growth in comparison with the control group. The calculated hepatic replacement area was 0.16% (95% CI, 0.1–0.22; \( P = 0.018 \), Student’s \( t \) test versus control group; Fig. 2A and B). Although single-agent 2C3 treatment caused a reduction of the hepatic tumor replacement area of 22.3%, this difference did not reach statistical significance. The calculated average was 0.37% (95% CI, 0.2–0.54; \( P = 0.348 \), Student’s \( t \) test versus control group). The highest suppression of metastatic tumor growth was observed in mice receiving the combination therapy. The average hepatic replacement area was 0.05% (95% CI, 0.04–0.06; \( P = 0.0053 \), Student’s \( t \) test versus control group). Furthermore, the combination therapy significantly improved the results achieved in both single agent-treated groups (\( P = 0.0099 \), Student’s \( t \) test for comparison between 2C3-treated
Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Tumor Cell Proliferation in Pancreatic Cancer. To determine whether the observed tumor growth suppression was caused by inhibition of cell proliferation, we investigated the effect of rapamycin alone and in combination with 2C3 on tumor cell proliferation as measured by BrdUrd incorporation with a BrdUrd-specific antibody. As shown in Fig. 3, the average amount of BrdUrd-labeled nuclei in 10 randomly selected microscopic fields was 493 (95% CI, 453.9–546.1) observed in the control group. A significant inhibition of tumor cell proliferation was found in the rapamycin-treated group. The average amount of proliferating cells was 380.2 (95% CI, 353.3–417.3; \( P = 0.0018 \), Student’s \( t \) test versus control group). No significant differences were observed in the 2C3-treated group by scoring 457.8 proliferating cells (95% CI, 432.4–497.9; \( P = 0.179 \), Student’s \( t \) test versus control group). However, the highest inhibition of tumor cell proliferation was seen in the combination therapy-treated group. An average of 270.8 (95% CI, 251.4–303.2) proliferating cells were counted (\( P < 0.0001 \), Student’s \( t \) test versus control group). The combination therapy showed a significantly stronger suppression of tumor cell proliferation in comparison with both single agent-treated groups (\( P < 0.0001 \), Student’s \( t \) test for comparison between combination therapy-treated group and 2C3-treated group; \( P = 0.0002 \), Student’s \( t \) test for comparison between combination therapy-treated group and rapamycin-treated group).

Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Apoptosis in Pancreatic Cancer. To further investigate the mechanism of the observed tumor-suppressive activities, we examined the effect of rapamycin alone and in combination with 2C3 on tumor cell apoptosis by TUNEL. The average number of TUNEL-positive cells measured in 10 randomly selected microscopic fields in the control group was 9.6 (95% CI, 8.2–11.1). The rapamycin-treated group showed a significant increase in the number of apoptotic cells. The calculated average was 18.7 (95% CI, 17.2–20.1; \( P = 0.0001 \), Student’s \( t \) test versus control group). A significant increase was also observed in the 2C3-treated group with an average of 24.7 apoptotic cells (95% CI, 20.8–28.5; \( P < 0.0001 \), Student’s \( t \) test versus control group) and in the combination therapy-treated group with 25 apoptotic cells (95% CI, 20.7–29.3; \( P = 0.0001 \), Student’s \( t \) test versus control group). No significant difference was observed between the 2C3-treated group and the group receiving the combination therapy \( (P = 0.815, \text{Student’s } t \text{ test for comparison between the } 2C3\text{-treated group and combination therapy-treated group}) \). The combination therapy did improve the results achieved in the rapamycin-treated group \( (P = 0.0039, \text{Student’s } t \text{ test for comparison between the rapamycin-treated group and combination therapy-treated group}) \). To confirm these results,
we also examined the distribution of apoptosis-inducing Fas receptor CD95. The average number of CD95-positive cells measured in 10 randomly selected microscopic fields was 74.3 (95% CI, 47.1–101.6) in the control group. A significant increase in the number of apoptotic cells was observed for the rapamycin-treated group with an average of 182.3 (95% CI, 120.6–247.4; \( P = 0.0024 \), Student’s \( t \) test versus control group). The combination therapy-treated group also showed a significant increase in the number of apoptotic cells with an average of 229 (95% CI, 207.5–250.5; \( P = 0.0024 \), Student’s \( t \) test versus control group). A very significant effect of rapamycin alone and in combination with 2C3 on apoptosis in pancreatic cancer \( \text{in vivo} \) cells. The control group received only carrier solution. *, \( P = 0.0018 \), Student’s \( t \) test versus control group; **, \( P < 0.0001 \), Student’s \( t \) test versus control group. No significant differences were observed in the 2C3-treated group (\( P = 0.179 \), Student’s \( t \) test versus control group). All statistical tests were two-sided. Error bars show 95% CIs.

**Effect of Rapamycin Alone and in Combination with Anti-Vascular Endothelial Growth Factor Antibody 2C3 on Tumor Angiogenesis in Pancreatic Cancer.** Because recent investigations suggest that rapamycin may have antiangiogenic activities linked to a decrease in production of VEGF and to a markedly inhibited response of vascular endothelial cells to stimulation by VEGF (6), we were interested in determining the effect of rapamycin alone and in combination with 2C3 on tumor angiogenesis. Therefore, we stained tumor sections with a rat antimouse monoclonal CD31 antibody. The average number of CD31-positive vessels measured in the control group was 626 (95% CI, 554.3–697). We found a significant decrease of tumor vessels measured in the rapamycin-treated group. The average number of stained vessels was 211.3 (95% CI, 188.3–234.4; \( P < 0.0001 \), Student’s \( t \) test versus control group). As expected, we also found a significant inhibition of tumor angiogenesis in the group treated with only 2C3. The average number of stained vessels was 252 (95% CI, −16.6 to 520.6; \( P = 0.0044 \), Student’s \( t \) test versus control group). An additional effect on tumor angiogenesis was observed in the group that received both drugs. The average number of stained blood vessels was 144.5 (100.03–188.9; \( P = 0.0002 \), Student’s \( t \) test versus control group). The combination therapy-treated group showed significantly fewer tumor vessels than the group treated with rapamycin alone (\( P = 0.0029 \), Student’s \( t \) test for comparison between the combination therapy-treated group and the rapamycin-treated group). No significant difference was found between the group treated with 2C3 and the group treated with combination therapy (\( P = 0.2747 \); Fig. 5).

**Fig. 3** Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on tumor cell proliferation in pancreatic cancer \( \text{in vivo} \). Cell proliferation in tumor tissue of SCID/beige mice that received BrdUrd by intraperitoneal injections. Mice were treated with 2C3, rapamycin, or rapamycin in combination with 2C3 after orthotopic injection of AsPC-1 human pancreatic cancer cells. The control group received carrier solution. *, \( P = 0.0018 \), Student’s \( t \) test versus control group; **, \( P < 0.0001 \), Student’s \( t \) test versus control group. No significant differences were observed in the 2C3-treated group (\( P = 0.179 \), Student’s \( t \) test versus control group). All statistical tests were two-sided. Error bars show 95% CIs.

**Fig. 4** Effect of rapamycin alone and in combination with anti-VEGF antibody 2C3 on apoptosis in pancreatic cancer \( \text{in vivo} \). SCID/beige mice were treated with 2C3, rapamycin, or rapamycin in combination with 2C3 after orthotopic injection of AsPC-1 human pancreatic cancer cells. The control group received only carrier solution. A. To detect tumor cell apoptosis, TUNEL was used. The average number of TUNEL-positive cells was scored in 10 randomly selected microscopic fields. *, \( P < 0.0001 \), Student’s \( t \) test versus control group; **, \( P < 0.0001 \), Student’s \( t \) test versus control group; ***, \( P < 0.0001 \), Student’s \( t \) test versus control group. No significant difference was observed between the 2C3-treated group and the group receiving the combination therapy (\( P = 0.815 \), Student’s \( t \) test). All statistical tests were two-sided. Error bars show 95% CIs. B. To determine the distribution of apoptosis inducing Fas receptor CD95, a monoclonal anti-Fas antibody was used. The average number of CD95-positive cells in 10 randomly selected microscopic fields was calculated. *, \( P = 0.0024 \), Student’s \( t \) test versus control group; **, \( P = 0.0023 \), Student’s \( t \) test versus control group; ***, \( P < 0.0001 \), Student’s \( t \) test versus control group. No significant difference was observed between the single-agent treatment groups and the group receiving the combination therapy. All statistical tests were two-sided. Error bars show 95% CIs.
DISCUSSION

Our data indicate that rapamycin, alone and in combination with antiangiogenesis therapy, strongly inhibits primary and metastatic tumor growth in pancreatic cancer in vivo. Furthermore, we could show that the combination therapy inhibits liver metastasis more assertively than treatment with a single agent. Although the difference between the primary tumor volume of mice treated with combination therapy versus rapamycin alone did not reach a statistical significance, we found an approximately 19% smaller pancreatic tumor volume in the combination therapy-treated group. However, to our knowledge, there are no in vivo studies thus far using rapamycin as a single treatment as well as rapamycin in combination with antiangiogenic therapy in this highly aggressive tumor model of pancreatic cancer.

To explain the potential antitumor effect of rapamycin alone and in combination with anti-VEGF antibody 2C3, we considered three basic theories. First, rapamycin may directly inhibit tumor cell proliferation. Grewe et al. (9) found evidence that rapamycin inhibits basal p70s6K activity and induced de-phosphorylation of p70s6K and 4E-BP1 in human pancreatic cancer lines MiaPaCa-2 and Panc-1. Rapamycin also inhibited DNA synthesis and anchorage-dependent and -independent proliferation. Finally, rapamycin strikingly inhibited cyclin D1 expression in pancreatic cancer cells (9). Additionally, Shah et al. (10) have shown that rapamycin significantly inhibited serum-induced proliferation in two human pancreatic cancer cell lines, BxPC3 and Panc-1. The inhibition observed in both cell lines was about 30%. Our in vivo results also strongly support this hypothesis because we found an inhibition of tumor cell proliferation of approximately 23% after single-agent rapamycin treatment. The single-agent treatment with 2C3 caused no significant inhibition of tumor cell proliferation. Surprisingly, the combination therapy did improve the results achieved with single-agent rapamycin treatment. The precise contribution of 2C3 to the observed improvement of the therapeutic effect of rapamycin is difficult to determine and is not yet clear to us.

From a rather mechanistic perspective, a possible explanation for this phenomenon could be the fact that VEGF-A induces not only angiogenesis but also lymphangiogenesis. Nagy et al. (11) could show that VEGF-A (VEGF_{164/165}) induces proliferation of lymphatic endothelium, resulting in the formation of greatly enlarged and poorly functioning lymphatic channels. They described “giant vessels” with incompetent valves, sluggish flow, and delayed lymph clearance (11). Consequently, the lymphatic malformation enhanced the VEGF-A–driven plasma extravasation and edema (11). This mechanism, in turn, could cause a poor biological availability of additional administered drugs. Using anti-VEGF antibody 2C3 might have improved the biological availability of rapamycin. Other authors support the hypothesis that after antiangiogenic treatment, the blood vessels that are still there will carry more blood to the tumor and in that way enhance the delivery of drugs (12). Further investigations will be necessary to elucidate the mechanism by which antiangiogenesis therapy improves drug effects when administered in a combination therapy.

Secondly, the observed antitumor effect of rapamycin alone and in combination with 2C3 may be a result of drug-induced apoptosis. Nave et al. (13) found that Akt/protein kinase B regulates the phosphorylation state of mTOR directly. Akt, an apoptotic regulator, is frequently activated in pancreatic cancer (14). Although rapamycin is not considered to be an antia apoptotic factor, a recent publication established Akt signaling through mTOR and eIF-4E as an important mechanism of oncogenesis and drug resistance in vivo by proving that rapamycin can restore apoptotic sensitivity to lymphomas expressing Akt in vivo (15). Our studies also indicate that rapamycin might have an effect on regulation of programmed cell death. We found an almost 2-fold induction of tumor cell apoptosis after single-agent rapamycin treatment. The observed induction of apoptosis by disrupting VEGFR2 signaling was not unexpected. It is known that targeting VEGF (16) or VEGFR (17) in pancreatic cancer in vivo causes a higher apoptosis index. Our results suggest that 2C3 as a single-agent treatment induces more apoptosis in pancreatic cancer than rapamycin as a single agent and that those effects are not additive.

The third theory about the observed antitumor effect of rapamycin alone and in combination with 2C3 is based on the fact that rapamycin showed antiangiogenic activities in vivo not only by decreasing the production of VEGF but also by inhibiting the response of vascular endothelial cells on stimulation by VEGF (6). Our findings strongly support this theory because we found a significant inhibition of tumor angiogenesis after single-agent rapamycin treatment. The effect of 2C3 on tumor angiogenesis was as expected. 2C3 is known to decrease the mean microvascular density in an orthotopic model of human breast cancer in nude mice (18). The combination therapy of rapamycin plus anti-VEGF antibody 2C3 amplified the inhibition of tumor angiogenesis from 66% after single-agent rapamycin treatment to 77% compared with the control.

One of the major problems (besides the rapid primary tumor progression in pancreatic cancer) is an early and wide-
spreading metastatic tumor growth. To address this issue, we were also interested in determining whether rapamycin treatment, alone or in combination with anti-VEGF antibody, has an impact on metastasis. We found significant suppression of metastatic tumor growth in both groups treated with rapamycin, either alone or in combination with anti-VEGF antibody 2C3. Furthermore, the combination therapy improved the results achieved in both single agent–treated groups remarkably. To our knowledge, there are no single or combination therapies for pancreatic cancer thus far achieving comparable results.

Hence, the combination therapy of rapamycin and anti-VEGF antibody 2C3 could be a new and promising therapeutic approach to the treatment of pancreatic cancer. The suppression of primary tumor volume and the significant reduction of metastasis in the combination therapy–treated group was caused by a combinatorial effect of both rapamycin and 2C3. Tumor cell proliferation and metastasis were predominantly regulated by rapamycin, although there were additive effects resulting from treatment with anti-VEGF antibody 2C3. On the other hand, 2C3 antibody plays a major role in regulating programmed cell death in the combination therapy–treated group. Both rapamycin and 2C3 antibody are important for inhibiting tumor angiogenesis.

Taken together, our studies propose new therapeutic strategies to inhibit both primary and metastatic tumor growth in pancreatic cancer. Rapamycin administered as a single agent as well as in combination with anti-VEGF antibody 2C3 strongly inhibits pancreatic tumor growth and liver metastasis. Nevertheless, the combination therapy has significant advantages compared with the single-agent treatment with rapamycin, especially when considering the fact that early metastatic tumor growth is a crucial problem in advanced stages of pancreatic cancer.

ACKNOWLEDGMENTS

We thank Dr. Steven King from Peregrine Pharma Inc. for the generous gift of anti-VEGF antibody.

REFERENCES

Clinical Cancer Research

Effect of Rapamycin Alone and in Combination with Antiangiogenesis Therapy in an Orthotopic Model of Human Pancreatic Cancer

Susann Stephan, Kaustubh Datta, Enfeng Wang, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/20/6993

Cited articles
This article cites 16 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/20/6993.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/10/20/6993.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.