Rapamycin-Induced Endothelial Cell Death and Tumor Vessel Thrombosis Potentiate Cytotoxic Therapy against Pancreatic Cancer

Christiane J. Bruns, Gudrun E. Koehl, Markus Guba, Maksim Yezhelyev, Markus Steinbauer, Hendrik Seeliger, Astrid Schwend, Anna Hoehn, Karl-Walter Jauch, and Edward K. Geissler

Department of Surgery, University of Regensburg, Regensburg, Germany

ABSTRACT

Purpose: Despite current chemotherapies, pancreatic cancer remains an uncontrollable, rapidly progressive disease. Here, we tested an approach combining a recently described antiangiogenic drug, rapamycin, with standard gemcitabine cytotoxic therapy on human pancreatic tumor growth.

Experimental Design: Tumor growth was assessed in rapamycin and gemcitabine-treated nude mice orthotopically injected with metastatic L3.6pl human pancreatic cancer cells. H&E staining was performed on tumors, along with Ki67 staining for cell proliferation and immunohistochemical terminal deoxynucleotidyl transferase-mediated nick end labeling and CD31 analysis. Rapamycin-treated tumor vessels were also directly examined in dorsal skin-fold chambers for blood flow after thrombosis induction. Cell death in human umbilical vein endothelial cells was assessed by flow cytometry after annexin-V staining.

Results: Rapamycin therapy alone inhibited tumor growth and metastasis more than gemcitabine, with remarkable long-term tumor control when the drugs were combined. Mechanistically, H&E analysis revealed tumor vessel endothelium damage and thrombosis with rapamycin treatment. Indeed, dorsal skin-fold chamber analysis of rapamycin-treated tumors showed an increased susceptibility of tumor-specific vessels to thrombosis. Furthermore, terminal deoxynucleotidyl transferase-mediated nick end labeling/CD31 double staining of orthotopic tumors demonstrated apoptotic endothelial cells with rapamycin treatment, which also occurred with human umbilical vein endothelial cells in vitro. In contrast, gemcitabine was not antiangiogenic and, despite its known cytotoxicity, did not reduce proliferation in orthotopic tumors; nevertheless, rapamycin did reduce tumor proliferation.

Conclusions: Our data suggest a novel mechanism whereby rapamycin targets pancreatic tumor endothelium for destruction and thrombosis. We propose that rapamycin-based vascular targeting not only reduces tumor vascularization, it decreases the number of proliferating tumor cells to be destroyed by gemcitabine, thus introducing a new, clinically feasible strategy against pancreatic cancer.

INTRODUCTION

Pancreatic cancer remains a major unsolved health problem with an estimated overall 5-year survival rate of only 1–4%, making it one of the leading causes of cancer-related mortality. Presently, over 80% of these patients have locally advanced or metastatic disease at the time of diagnosis, which excludes even the possibility of curative surgery (1–3). Moreover, tumor control in these cases is not normally successful with currently available systemic chemotherapy. In fact, a response rate of one-quarter or less can be expected with standard chemotherapy, with a dismal median survival of <6 months (4, 5). With this background, the question is what different approach, besides standard cytotoxic therapy, could be used to attack this aggressive, highly resistant form of cancer. One key to this question could lie in the emerging realization that pancreatic tumors may be susceptible to antiangiogenic therapy (6–9).

Indeed, recent clinical studies suggest that pancreatic cancer is highly angiogenesis dependent. More specifically, clinical prognostic data indicate that expression of proangiogenic factors such as vascular endothelial growth factor (VEGF), epidermal growth factor, and thymidine phosphorylase positively correlates with a higher relapse rate and shorter patient survival (10, 11). Furthermore, a high density of microvessels within pancreatic tumors is a prognostic factor for early disease progression (10, 12, 13). Therefore, we hypothesized in the current study that pancreatic cancer progression may be sensitive to antiangiogenic therapy, particularly when combined with a cytotoxic agent. With regard to antiangiogenic therapy, we chose to test whether the mammalian target of rapamycin inhibitor rapamycin could be effective against metastasizing pancreatic cancer. This choice was based on our recent study showing that rapamycin is a potent antiangiogenic substance, working most effectively at nontoxic, nanomolar concentrations (14). The antiangiogenic activity of rapamycin is due, at least in part, to...
inhibition of VEGF production and blockade of VEGF-mediated stimulation of endothelial cells. However, a clinically relevant corollary to this initial study was that nests of tumor cells not requiring angiogenesis continued to exist and eventually progressed into larger masses once the rapamycin therapy was discontinued. Therefore, in the present study, we tested the possibility that the combination of cytotoxic chemotherapy with rapamycin could better control or reduce these nests of tumor cells over a long-term period. In the situation of pancreatic carcinoma, our approach combines daily rapamycin treatment with repeated use of the best available cytotoxic drug for this disease, gemcitabine. Mechanistically, intracellular phosphorylation of gemcitabine produces di- and triphosphate molecular forms capable of acting as a fraudulent base in DNA and inhibiting DNA synthesis-dependent ribonucleotide reductase (15), together producing a strong cytotoxic effect.

Using a model of metastatic human pancreatic cancer in nude mice, our present study shows that antiangiogenic therapy with rapamycin alone has an antitumor effect exceeding that of gemcitabine and that the combination of rapamycin and gemcitabine dramatically reduces long-term tumor growth and the development of metastases. Mechanistically, our data suggest that rapamycin affects tumor vascularization and decreases the number of proliferating tumor cells, thereby enhancing the effectiveness of gemcitabine’s cytotoxic activity against tumor growth. Moreover, this study provides the first evidence that tumor control achieved with rapamycin is associated with tumor progression, the tumor mass was held between the fingers and moved to the abdominal surface, where its size could be measured using a caliper. Tumor volume was estimated by the formula

\[ V = \pi/6(a \times b \times c), \]

where \( a, b, \) and \( c \) represent the length, width, and height of the mass. For H&E staining and immunohistochemical analysis, half of the primary tumor was fixed in formalin for paraffin embedding, and the other half was prepared for frozen sectioning. Metastatic L3.6pl tumor growth was also evaluated. For metastases in the liver, macroscopically visible tumor nodules (\( \geq 1 \) mm) were noted on the liver surface. Furthermore, enlarged regional (celiac and para-aortic) lymph nodes were recorded. Liver and lymph node tissue were excised and processed to confirm metastases by H&E staining.

In one experiment, all mice in the control group and 6 of 10 mice from each treatment group were sacrificed as usual on day 28 after orthotopic tumor cell injection. The pancreatic tumor and metastases were analyzed as described above. However, the remaining four mice in each treatment group were kept alive to obtain long-term data, and drug therapy was continued. Those mice in good condition were kept alive until day 60; any mice showing progressive tumor growth, signs of tumor burden, drug toxicity (weight loss \( \geq 20\% \)), or reduction in mobility to easily access food and water were sacrificed. To monitor cancer progression, the tumor mass was held between the fingers and moved to the abdominal surface, where its size could be measured using a caliper. Tumor volume was estimated by the formula

\[ V = \pi/6(a^2 \times b), \]

where \( a \) is the width of the tumor, and \( b \) is the length of the tumor.

**Materials and Methods**

**Pancreatic Cancer Model and Treatment Regimens.** The highly metastatic human pancreatic cancer cell line L3.6pl was maintained in cultures supplemented as described previously (16). Using animal procedures approved by the local authorities, \( 1 \times 10^5 \) L3.6pl tumor cells were orthotopically implanted in the subcapsular region of the pancreas of male athymic 8–12-week-old nude mice (BALB/c nu/nu; Charles River, Sulzfeld, Germany), as detailed previously (16). After implantation, tumors were allowed to grow for 7 days before treatment initiation. At the start of treatment, the median tumor volume in sacrificed mice is typically 18 mm\(^3\) (17). Tumor-bearing mice were randomized and subjected to the following treatment:

- (a) 1.5 mg/kg/day rapamycin (5 mg/ml stock solution; Wyeth Pharma, Münster, Germany) by i.p. injection;
- (b) biweekly 50 or 100 mg/kg gemcitabine (Gemzar 1000 powder dissolved in 0.9% saline; Lilly, Giessen, Germany) by i.p. injection;
- (c) i.p. combination of 1.5 mg/kg/day rapamycin with either 50 or 100 mg/kg gemcitabine biweekly; or
- (d) i.p. injections of 0.9% saline control solution at corresponding time points (rapamycin and gemcitabine were diluted for injection with 0.9% saline).

Mice were sacrificed on day 28 after tumor cell injection in experiments aimed at measuring tumor growth at a fixed point. Excised pancreatic tumors were weighed and measured. The tumor volume was then calculated using the formula

\[ V = \pi/6(a \times b \times c), \]

where \( a, b, \) and \( c \) represent the length, width, and height of the mass. For H&E staining and immunohistochemical analysis, half of the primary tumor was fixed in formalin for paraffin embedding, and the other half was prepared for frozen sectioning. Metastatic L3.6pl tumor growth was also evaluated. For metastases in the liver, macroscopically visible tumor nodules (\( \geq 1 \) mm) were noted on the liver surface. Furthermore, enlarged regional (celiac and para-aortic) lymph nodes were recorded. Liver and lymph node tissue were excised and processed to confirm metastases by H&E staining.

In one experiment, all mice in the control group and 6 of 10 mice from each treatment group were sacrificed as usual on day 28 after orthotopic tumor cell injection. The pancreatic tumor and metastases were analyzed as described above. However, the remaining four mice in each treatment group were kept alive to obtain long-term data, and drug therapy was continued. Those mice in good condition were kept alive until day 60; any mice showing progressive tumor growth, signs of tumor burden, drug toxicity (weight loss \( \geq 20\% \)), or reduction in mobility to easily access food and water were sacrificed. To monitor cancer progression, the tumor mass was held between the fingers and moved to the abdominal surface, where its size could be measured using a caliper. Tumor volume was estimated by the formula

\[ V = \pi/6(a^2 \times b), \]

where \( a \) is the width of the tumor, and \( b \) is the length of the tumor.

**Immunohistochemical Staining for Ki67, Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL), and CD31.** Cell proliferation analysis was performed on paraffin-embedded tissues with standard Ki67 staining techniques (18, 19). Briefly, a mouse antihuman Ki67 monoclonal antibody (DAKO A/S, Glostrup, Denmark) was used in the primary reaction. The DAKO EnVision System, containing a secondary horseradish peroxidase-conjugated antipan smooth muscle actin complex, was used with 3,3'-diaminobenzidine to detect Ki67. Sections were counterstained with Gill’s hematoxylin. To quantify the amount of proliferation, all Ki67-positive and -negative cells were counted in 10 random high-power fields (0.159 mm\(^2\) at \( \times 100\) magnification) per slide.

Colorimetric immunohistochemical staining for apoptotic cell death (TUNEL) was performed on paraffin-embedded tissue sections using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) and the AEC substrate pack (Biogenex, Hamburg, Germany), according to the manufacturers’ instructions.

Analysis of apoptotic endothelial cells was performed on frozen tissue sections using a previously described immunofluorescent CD31/TUNEL double-labeling technique (17). Briefly, sections were first incubated with a rat antimouse CD31/platelet/endothelial cell adhesion molecule 1 monoclonal antibody (PharMingen, San Diego, CA), followed by staining with Texas red-conjugated goat antirat IgG (Jackson ImmunoResearch Laboratories, West Grove, CA). A TUNEL procedure was subsequently performed using the Fluorescein Apoptosis Detection System (Promega, Madison, WI).

**Dorsal Skin-Fold Chamber (DSFC) Analysis.** Tumor angiogenesis was analyzed in vivo via the transparent DSFC model, as described previously (20, 21). Chambers were inoculated with \( 1 \times 10^5 \) L3.6pl cells. The day after tumor inoculation, mice were treated i.p. with saline or 1.5 mg/kg/day rapa-
mycin. On day 7, intravital microscopy (Zeiss Axiootech Vario microscope; Göttingen, Germany) was performed on DSFCs to examine tumor blood vessels. The entire tumor was examined, and these images (7–15 images/tumor) were recorded on video for analysis (modified Sony 3CCD Color Video Camera; AVT Horn, Aalen, Germany). Vessel diameter was measured using Image J software (from Wayne Rasband; Version 1.25s; NIH, Bethesda, MD) by generating horizontal grid lines every 50 pixels. Tumor vessels crossing the grid lines were individually measured, whereas vertically aligned vessels were not included in the analysis.

Blood flow in tumor vessels in DSFCs was measured directly using a modified thrombosis induction technique (22). In principle, i.v. injected FITC-dextran (M, 464,000; Sigma-Aldrich Chemicals, St. Louis, MO), when activated by prolonged UV light irradiation, causes oxidative stress by free-radical production as well as activation of the thrombosis cascade (22). In our experiments, L3.6pl tumors were allowed to grow in DSFCs of nude mice for 7 days, with or without rapamycin treatment (1.5 mg/kg/day). Mice then received injection via the tail vein with 0.5 ml of 5% FITC-dextran dissolved in PBS. At the same time, mice also received i.v. injection with 8 × 10⁷ red blood cells that had been labeled with a red fluorescent stain (Red Fluorescent Cell Linker Kit; Sigma-Aldrich Chemicals). The fluorescent red blood cells could be easily seen flowing through blood vessels in the tumors of the DSFCs by intravital microscopy. Phototoxic UV (Zeiss filter set EX BP 450–490, BSFT 510, EM BP 515–565) light was directly applied to a vascular area of the tumor through a ×20 objective lens for 1 min, resulting in a dose of 1010 mW/cm². Then, the vascular architecture was observed for 30 s under normal bright-field light, followed by 30 s of RBC flow observation under filtered light for red fluorescence (Zeiss filter set EX BP 546/12, BS FT 580, EM LP 590). The cycle of phototoxic, bright-field, and red fluorescent light was repeated up to a maximum of 20 times. When all of the blood vessels within the area showed total occlusion (no blood flow), this time point was recorded, and light cycles were discontinued. In addition, normal vascular areas clearly outside the tumor region were analyzed in the same way.

**In Vitro Cell Proliferation Assay.** L3.6pl cells were cultured for 48 h in 96-well microtiter plates in medium with or without rapamycin or gemcitabine. Proliferation was assessed by adding bromodeoxyuridine (bromodeoxyuridine proliferation kit; Roche Diagnostics GmbH, Mannheim, Germany) to individual wells 4 h before completion of the 48-h incubation period and then measuring absorbance at 450 nm.

**Fluorescence-Activated Cell-Sorting Analysis for Cell Death.** Human umbilical vein endothelial cells (HUVECs) were cultured under normal conditions with endothelial cell basal medium (PromoCell, Heidelberg, Germany) supplemented with growth factors (PromoCell) and 2% fetal bovine serum, or they were placed under minimal culture conditions, where cells were deprived of fetal bovine serum and other supplements. Under supplement and serum-deprived conditions, recombinant human VEGF₁₆₅ (R&D Systems, Wiesbaden, Germany) was added at a concentration of 50 ng/ml in the presence of increasing concentrations of rapamycin. After 8 h, HUVECs were removed from the culture dishes with gentle trypsinization, labeled with annexin V-FITC (R&D Systems), and analyzed by flow cytometry.

**Statistical Analysis.** Data are given as the mean ± SEM in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student’s t test was performed with InStat 3.0 Statistical Software (Graphpad Software, San Diego, CA).

### RESULTS

**Growth and Metastasis of Established Pancreatic Tumors.** To determine the potential for rapamycin treatment in a pancreatic cancer situation, athymic nude mice received orthotopic injection with metastatic human L3.6pl cancer cells. Pancreatic tumors were allowed to become established for 7 days before initiation of rapamycin or gemcitabine treatment. Standard doses of rapamycin (1.5 mg/kg/day) and gemcitabine (100 mg/kg, 2×/week) were used in the first group of experiments, and all animals were sacrificed 28 days after tumor cell injection. All control mice and treated animals did develop primary pancreatic tumors, but the growth and extent of tumor progression depended on the treatment regimen. Standard pancreatic cancer treatment with gemcitabine alone resulted in a significant reduction in the pancreatic tumor volume, compared with control mice (Table 1). Interestingly, and unexpectedly, rapamycin treatment alone reduced tumor volume 2-fold more than standard gemcitabine therapy. Furthermore, when rapamycin and gemcitabine treatment were combined, tumors were very small, growing to only 19% of the size observed with gemcitabine treatment alone. Mice tolerated rapamycin well (0.8 ± 1.6% weight gain), with animals treated with gemcitabine alone or

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Orthotopic pancreatic tumor</th>
<th>Metastases (incidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Volume (mm³)</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>8/8</td>
<td>1672 ± 144</td>
</tr>
<tr>
<td>Gemcitabine (100 mg/kg, 2×/week)</td>
<td>9/9</td>
<td>773 ± 117²</td>
</tr>
<tr>
<td>Rapamycin (1.5 mg/kg/day)</td>
<td>10/10</td>
<td>388 ± 5³,⁴</td>
</tr>
<tr>
<td>Rapamycin + gemcitabine</td>
<td>9/9</td>
<td>147 ± 23²,⁴,⁵</td>
</tr>
</tbody>
</table>

*² P < 0.00002 versus saline-injected controls.
*³ P < 0.01 versus gemcitabine.
*⁴ P < 0.001 comparing rapamycin + gemcitabine treatment versus rapamycin alone.

**Table 1** Effect of rapamycin and gemcitabine on established human L3.6pl pancreatic tumors in nude mice

All values were obtained from mice sacrificed 28 days after orthotopic tumor cell injection.
Rapamycin-induced thrombosis targets pancreatic cancer

Metastasis of pancreatic tumors was also affected by the different treatment regimens. Lymph node metastases were reduced by rapamycin treatment and completely eliminated by combination therapy with rapamycin and gemcitabine, but treatment with gemcitabine alone did not reduce the incidence of these metastases (Table 1). On day 28, macroscopically visible liver metastases were present in 50% of controls, and this tended to be reduced in frequency by all three treatment regimens, with combination therapy giving the lowest incidence.

A second group of similar experiments was performed to test whether a lower dose of gemcitabine could be effective and sustained long-term. Results showed that in mice sacrificed at 28 days, low-dose gemcitabine (50 mg/kg) inhibited tumor growth to the same degree as the higher dose (100 mg/kg; Fig. 1A). However, combination therapy using high-dose gemcitabine combined with rapamycin did lead to a slightly greater reduction in tumor volume, compared with the rapamycin combination with low-dose gemcitabine (P < 0.001). In these same experiments, all controls were sacrificed on day 28 because of their deteriorating condition, but 4 of 10 drug-treated mice were continued on therapy for as long as 60 days to determine long-term effects (Fig. 1B). All mice on single-agent therapy or high-dose rapamycin + gemcitabine had to be sacrificed by day 53 because of either tumor progression or therapy side effects. In contrast, all mice on low-dose gemcitabine + rapamycin therapy tolerated the treatment well and survived throughout the observation period. Moreover, this treatment group showed an average total weight loss of <10% at day 60; between day 28 and day 60, animal weight remained quite stable in this group (weight loss < 5%). Importantly, tumor growth estimations made by in vivo palpation measurements showed that the pancreatic tumor volume remained stable in these mice between day 40 (211 ± 49 mm³) and day 60 (218 ± 54 mm³), which is also nearly identical to measurements made in sacrificed animals from the same group on day 28 (Fig. 1A).

Analysis of Pancreatic Tumors for Proliferation. Ki67 staining for cell proliferation was performed in the tumors removed from the animals on day 28. Results from this analysis show that the relative number of Ki67-positive tumor cells was substantially less in tumors from mice treated with rapamycin or rapamycin + gemcitabine, when compared with control tumors (Fig. 2, A and B). In contrast, gemcitabine had no significant effect on tumor cell proliferation compared with controls. Interestingly, results from in vitro pancreatic tumor cell proliferation assays did not completely reflect what was observed directly in the tumors. More specifically, rapamycin at concentrations relevant in vivo did show some antiproliferative effect on cultured L3.6pl tumor cells, but gemcitabine also demonstrated an antiproliferative effect, albeit at relatively high concentrations (Fig. 2C). Notably, cytotoxic effects with gemcitabine are typically seen in the micromolar range with pancreatic cancer cells (23); therefore, the decrease in proliferation in this assay may be due at least in part to a reduction in cell numbers. In contrast, rapamycin in the concentrations tested is not cytotoxic to L3.6pl cells (data not shown), suggesting some direct antiproliferative effect.

Histomorphological Analysis of Pancreatic Tumors after Therapy. Standard H&E and TUNEL staining of tumors removed after 28 days revealed some striking features with regard to blood vessel formation in rapamycin-treated mice. In rapamycin-treated or rapamycin + gemcitabine-treated tumors, we consistently observed the presence of dilated tumor vessels with thrombosis, destruction and detachment of the endothelial cell layer were observed. The pathological effects of the thrombosis could be seen by the death of tumor cells in the areas surrounding the incapacitated vessels (Fig. 3B).
These perivascular tumor cells exhibited signs of apoptosis evident from the accumulation of apoptotic bodies within the dying cells. Importantly, neither tumors from controls nor tumors from mice treated with gemcitabine alone showed any of the same signs of vascular thrombosis (Fig. 3A). Furthermore, there were no signs of thrombosis within vessels of adjacent normal pancreatic tissue in rapamycin-treated mice, suggesting that the thrombotic effect was localized to the tumor (Fig. 3C).

To directly test the effect of rapamycin on tumor blood vessel flow dynamics, L3.6pl tumor cells were implanted into DSFCs, and vessels were examined by intravital microscopy on day 7. Results showed that tumor vessels in rapamycin-treated mice were dilated in size compared with tumor vessels in control mice (Fig. 4, A and B). Furthermore, when we photodynamically promoted vascular thrombosis in tumors, blood flow was rapidly blocked by thrombosis in rapamycin-treated mice, compared with tumor vessels in controls (Fig. 4C). Other comparisons indicate that tumor vessels in two of three control mice did show blood flow blockade slightly before that seen in nontumor vessels (area outside the tumor) of the same mice. Analysis of blood flow and thrombosis in nontumor vessels revealed that normal vessels in rapamycin-treated mice were less susceptible to thrombosis than tumor vessels, suggesting that rapamycin preferentially provokes clotting in tumor vessels. When comparing thrombosis only between nontumor vessels in controls and those in rapamycin-treated mice, flow continued in all controls for >20 min, whereas blood flow in nontumor areas in rapamycin-treated mice stopped between 17 and 19 min, indicating only a slightly increased rate of induced thrombosis in nontumor vessels with rapamycin treatment. Furthermore, to exclude the influence of tumor on thrombosis, thrombosis induction was performed in DSFCs without L3.6pl tumor implantation. In both the control and rapamycin-treated mice (n = 4 mice/group), thrombosis time exceeded 20 min (data not shown), suggesting that clot formation in blood vessels completely unassociated with tumor is not affected by rapamycin. Therefore, in general, tumor blood vessels in rapamycin-treated mice showed a pattern of development and function that was consistent with our histological vascular findings in the orthotopic pancreatic tumors.

**Effects on Endothelial Cell Survival and Proliferation.** Disruption of the tumor vessel endothelium observed in the earlier histological studies suggested that endothelial cells may be susceptible to cell death in the presence of rapamycin. To test this hypothesis, we first examined tumor vessels from rapamycin-treated mice for endothelial cell death by colorimetric TUNEL staining. Results showed that endothelial cells in these rapamycin-treated tumors, particularly in damaged and clotted vessels, were indeed positive for TUNEL (Fig. 5A). Tumor vessels from mice treated with rapamycin + gemcitabine also demonstrated a high rate of endothelial cell death. Next we performed a fluorescence double-labeling procedure for endo-
Fig. 3  Rapamycin treatment results in the development of thrombi in pancreatic tumors. A, increasing magnification views of an area of pancreatic tumor from control, rapamycin, gemcitabine, and combination treatment mice are shown. No thrombi were found in either control or gemcitabine-treated mice; however, the presence of thrombi was a predominant feature in tumors of mice treated with rapamycin alone or with rapamycin in combination with gemcitabine (×100, ×200). At ×400 magnification, normal blood vessels can be seen in control and gemcitabine-treated mouse tumors, whereas clotted tumor vessels in rapamycin-treated mice show disruption of the endothelial layer (arrows). B, this photomicrograph shows a clotted vessel in a rapamycin + gemcitabine-treated tumor (arrow). Note the signs of tumor cell death (apoptotic bodies) in the area surrounding the thrombosed vessel. C, areas of normal pancreatic tissue outside tumors treated with rapamycin (+gemcitabine) showed no evidence of vessel thrombosis (arrows). All bars in this figure = 50 μm.
thelial cells (CD31) and TUNEL, and we observed that structures in pancreatic tumors composed of endothelial cells could also be shown to be TUNEL positive (Fig. 5B). In contrast, endothelial cells in gemcitabine-treated tumors were not found to be TUNEL positive. To further test whether rapamycin causes endothelial cell death, we cultured HUVECs in the presence or absence of increasing concentrations of drug and performed flow cytometric analysis on annexin-FITC-labeled cells. Results showed that compared with full stimulation of HUVECs, serum and supplement-deprived conditions caused the cells to die (Fig. 6). Addition of VEGF to the serum and supplement-deprived environment rescued the cells from death. Interestingly, 5 nM rapamycin completely blocked the rescue effect of VEGF on HUVECs; lower drug levels had a lesser effect. Treatment of HUVECs with gemcitabine had no effect on annexin staining (data not shown). Together, these results suggest that endothelial cell survival pathways are blocked by rapamycin, but not by gemcitabine, leading to cell death and subsequent vascular endothelium disruption in tumor vessels.

DISCUSSION

Pancreatic cancer remains one of the most uncontrollable neoplasms encountered in clinical oncology. In this study, we present data that suggest human pancreatic cancer may be particularly responsive to a new therapeutic approach involving conventional use of the cytotoxic agent gemcitabine with a recently discovered antiangiogenic drug, rapamycin (14). Our study indicates that rapamycin use alone can inhibit human pancreatic tumor growth in nude mice to a greater degree than gemcitabine, a standard first-line chemotherapeutic agent available for the treatment of human pancreatic cancer. Moreover,
when gemcitabine was combined therapeutically with rapamycin, growth of established pancreatic tumors was severely compromised, and importantly, metastases to the liver and local lymph nodes were reduced or eliminated. The clinical promise and scope of the combined effects of rapamycin and gemcitabine were shown by the lack of tumor progression in long-term surviving nude mice bearing the human pancreatic cancer.

One of the most intriguing features of rapamycin treatment was the presence of damaged, dilated vessels containing thromboses in orthotopic pancreatic tumors. These areas of thrombosis were clearly associated with tumor attrition in regions surrounding the damaged vessels, thus restraining pancreatic cancer advancement. A closer look at the tumor vessel endothelium in rapamycin-treated mice revealed a potential cause for the thrombosis. More specifically, histological analysis showed damaged and sometimes detached endothelial cell layers that could also be shown to contain a high number of endothelial cells that had undergone cell death. Under more well-defined in vitro experimental conditions usingHUVECs, we could confirm that endothelial cells maintained with VEGF did not survive in the presence of rapamycin in the 0.1 nM range, with a maximal effect reached at 5 nM. Interestingly, data in the recent literature indirectly support the hypothesis that rapamycin treatment can induce apoptosis of VEGF-stimulated endothelial cells, potentially leading to tumor vessel thrombosis. The evidence begins with data indicating that VEGF induction of the phosphatidylinositol 3'-kinase/Akt intracellular signaling pathway is important for endothelial cell survival (24, 25). Phosphatidylinositol 3'-kinase/Akt up-regulation of FLICE-inhibitory protein protects endothelial cells from Fas-mediated apoptosis (25), which is critical because Fas is constitutively expressed on endothelial cells. It has also been shown that phosphatidylinositol 3'-kinase/Akt signaling inhibits endothelial cell death by down-regulating p38 mitogen-activated protein kinase-dependent apoptosis pathways (26). Therefore, it is logical to suggest that rapamycin inhibition of mammalian target of rapamycin, which is downstream of phosphatidylinositol 3'-kinase/Akt (27), could indeed be effective at inducing apoptosis of endothelial cells. Clinical observations also correlate with this hypothesis. For example, abnormal thrombus formation in microvascular thrombotic diseases such as idiopathic thrombocytopenic purpura has been linked to induction of endothelial cell apoptosis by soluble serum factors (28–30), and interestingly, thrombocytopenia and hemolytic uremic syndrome have been reported as
common side effects when rapamycin is used for immunosuppressive treatment of acute graft-versus-host disease (31). From another perspective, research on microangiopathic hemolytic diseases has revealed that endothelial cells derived from various tissues have different susceptibilities to apoptosis and thus to thrombosis. Because this variability has been linked to the relative tissue expression of several apoptosis survival genes, including Bcl-2-family genes and VEGF (28), the histological presence of thrombi in our study in the pancreatic tumors, but not in the surrounding normal pancreas, could be attributed to a differential expression pattern between the normal and cancerous tissue. Another contributing factor to the specificity of the thrombosis in the pancreatic tumors could be related to observations that pancreatic cancer patients tend to develop regional blood clots, reportedly due to thrombin activation (32). Indeed, our thrombus induction experiments in DSCFs support this observation but, most importantly, are the first to show that tumor vessels are particularly susceptible to thrombus formation with rapamycin treatment. Thus, rapamycin’s negative effect on the survival of rapidly expanding tumor-associated endothelial cells, combined with the reported local activation of thrombin via cancer cells, may favorably concentrate thrombotic events within pancreatic tumors.

Interestingly, from a completely different clinical perspective, our thrombosis induction experiments caution that nontumor vessels may exhibit some risk for clotting with rapamycin treatment (Fig. 4C). This could be of considerable significance because of the widespread use of rapamycin as an immunosuppressant in organ transplant patients. As mentioned previously, microangiopathic thrombosis can lead to severe side effects that have necessitated the discontinuation of this immunosuppressive treatment in some bone marrow transplant patients (31). The development of potentially fatal hepatic vein or artery thrombosis after rapamycin treatment following liver transplantation (33) further emphasizes possible effects of this drug on blood vessels and coagulation. In these instances, however, it is possible that blood vessel damage resulting from graft-versus-host reactions, immunological rejection, or surgical trauma raises the likelihood that rapamycin treatment could contribute to a thrombotic event. Therefore, there is a broad-based clinical need to understand the potential local specificity and mechanism of the prothrombotic effect of rapamycin in noncancerous tissue, as well as in tumors.

Nothwithstanding the potential importance of thrombosis, other mechanistic issues from our study relate to the question of how combined rapamycin and gemcitabine treatment keeps aggressive pancreatic tumors from advancing. Although the mechanisms are not clear, it is reasonable to speculate that the different activities of the two drugs strike at multiple critical aspects of tumor growth. One logical explanation for their combined potency could relate first to the ability of rapamycin to prevent vascular expansion and to promote tumor vessel thrombosis. However, whereas the present study suggests that these rapamycin effects alone clearly inhibit tumor growth, tumors do continue to expand slowly, leading to only a moderate improvement in long-term results (Fig. 1). Therefore, we reason that because rapamycin is not generally cytotoxic to tumor cells at the doses we used (34) and has only a moderate direct antiproliferative effect on pancreatic tumor cells in vitro, pockets of cells with at least some rudimentary angiogenesis can proliferate. The role of gemcitabine at this phase could then be to destroy tumor cells that do enter the S-phase of cell proliferation, which is one its primary anticancer activities (35). Interestingly, under circumstances where rapamycin is not present, gemcitabine’s cytotoxic effect alone is not able to completely counterbalance the concomitant high proliferation rate we observed in pancreatic tumors (Fig. 2A). Indeed, a lowering of the proliferation rate in pancreatic tumors was only associated with rapamycin treatment. As discussed previously, rapamycin did have some direct antiproliferative effect on L3.6pl cells in vitro, as has been reported with other pancreatic cell lines (36), but this may not be the only explanation for its exceptional antiproliferative activity in tumors. We suggest that there is an indirect antiproliferative effect of rapamycin correlating with its antiangiogenic activity and the previously reported observation that proliferation rate is inversely proportional to the distance of tumor cells from the nearest blood vessel (37, 38). Considering these data together, we propose that whereas rapamycin’s antiangiogenic, prothrombotic, and antiproliferative effects can reduce pancreatic tumor growth, an equally important “trap” must be set (i.e., gemcitabine) for those tumor cells that do acquire adequate resources to proliferate and advance tumor growth.

Consistent with this strategy, long-term control of pancreatic cancer progression in our experiments could only be achieved by combining rapamycin and gemcitabine.

Finally, clinical use of rapamycin in a gemcitabine-based protocol to treat human cancer is highly feasible. Currently,
rapamycin is approved for use in human organ transplantation as an immunosuppressive agent to prevent allograft rejection. The drug is maintained on a daily basis in patients for several years or indefinitely. An important corollary to this issue from our study is that rapamycin exerts its most potent effect on endothelial cells near 5 nM, which coincides with serum drug levels targeted in transplant patients. Therefore, it is reasonable to suggest that long-term, continuous inhibition of tumor neoangiogenesis is possible by incorporating these already thoroughly tested rapamycin treatment protocols into cancer treatment regimens. Another positive aspect of combining rapamycin with gemcitabine is that the latter agent can also be effectively and safely administered over an extended period at a reduced dose (39), lending credibility to the potential of a clinical protocol for long-term tumor control, as we were able to achieve in mice with low-dose gemcitabine + rapamycin treatment. Therefore, our study suggests that rapamycin and gemcitabine could offer a novel, clinically feasible drug therapy to control pancreatic cancer disease progression, and the general strategy of combining rapamycin with other cytotoxic drugs may also prove to be effective for a broader range of cancers for which drug cytotoxicity alone is not curative or does not provide tumor control with a favorable quality of life.

ACKNOWLEDGMENTS
We thank Dr. Hagen Blaszyk (Department of Pathology, University of Regensburg) for review of histological work presented in this paper. We also thank Christine Wagner for excellent technical assistance on the project.

REFERENCES
11. Baker CH, Solorzano CC, Fidler IJ. Blockade of vascular endothelial growth factor receptor and epidermal growth factor receptor signal-


Rapamycin-Induced Endothelial Cell Death and Tumor Vessel Thrombosis Potentiate Cytotoxic Therapy against Pancreatic Cancer

Christiane J. Bruns, Gudrun E. Koehl, Markus Guba, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/6/2109

Cited articles
This article cites 39 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/6/2109.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/6/2109.full#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.