Endothelial Akt Signaling Is Rate-Limiting for Rapamycin Inhibition of Mouse Mammary Tumor Progression

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Abstract

Chronic activation of Akt signaling in the endothelium recapitulates the salient features of a tumor vasculature and can be inhibited by rapamycin, an inhibitor of mammalian target of rapamycin. This led to the hypothesis that the antitumor efficacy of rapamycin may be partially dependent on its ability to inhibit endothelial Akt signaling. In this study, we determined whether Akt activation in the endothelium was rate-limiting for this antitumor response. We engineered mouse mammary tumor virus–polyoma virus middle T antigen mice to express myristoylated Akt (myrAkt) in endothelial cells under tetracycline control. Untreated DTG controls (n = 5) were compared to those used clinically in transplant patients, we observed strong inhibition of mammary tumor growth. To test whether Akt activation in the endothelium was rate-limiting for this antitumor response, we observed that primary human endothelial cells and fibroblasts showed that primary human endothelial cells and fibroblasts had a bimodal Akt response with effective reductions in phosphorylated Akt (pAkt) at 10 ng/mL. In contrast, rapamycin increased pAkt levels in tumor cell lines. Together, these data support the hypothesis that primary human endothelial cells and fibroblasts showed that primary human endothelial cells and fibroblasts had a bimodal Akt response with effective reductions in phosphorylated Akt (pAkt) treatment. Rapamycin did not affect pAkt levels in tumor cell lines. Together, these data support the hypothesis that endothelial Akt signaling plays an important role in the antitumor efficacy of rapamycin.

Materials and Methods

Cell culture. Human dermal microvascular endothelial cells and fibroblasts were isolated as previously described (10). These cells were cultured in EGM-2 media supplemented with 5% FCS and growth factors (Clonetics). MCF7 cells (from the cell line repository at the Lombardi Cancer Center, Georgetown University) were cultured in MEM supplemented with 10% FCS, 0.1 mmol/L of nonessential amino acids, 1 mmol/L of sodium pyruvate, and 0.01 mg/mL of insulin. Primary human mammary fibroblasts were isolated as described (11). These cells were cultured in DMEM supplemented with 10% calf serum. Use of human tissue was approved by the Beth Israel Deaconess Medical Center Institutional Review Boards. The transgenic mouse model that expresses constitutively active myristoylated Akt (myrAkt) in endothelial cells under tetracycline control has been previously described (9, 12). Control double transgenic mice (DTG) used in this study carried the mouse mammary tumor virus–polyoma virus middle T antigen (MMTV-PyT) transgene and either the VE-cadherin/TTA or the TET/myrAkt transgine. DTG mice developed mammary tumors but did not express endothelial cell myrAkt. Triple transgenic mice (TTG) carry all three transgenes MMTV-PyT, VE-Cadherin/TTA, and TET/myrAkt. These animals develop mammary tumors and express tetracycline-repressible myrAkt in endothelial cells. Untreated DTG controls (n = 5) were compared with rapamycin-treated DTG and TTG animals (n = 3–4 animals for each genotype and for each dose). All studies were conducted in compliance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee guidelines.

Tumor growth. MMTV-PyT virus–tumor–bearing female mice were taken off tetracycline to turn on myrAkt expression when tumors first became palpable, and on the same day treatment with rapamycin was initiated. Mice
were injected i.p. with rapamycin at the indicated doses everyday, and tumor growth was measured daily with a caliper. All tumor treatments were initiated at similar tumor sizes (i.e., when a tumor first became palpable). Tumor volume was calculated as volume = length \times width \times depth. Rapamycin (LC Laboratories) was prepared in a solvent as previously described (9). Means were calculated for each time point and graphed showing error bars for SDs. Unpaired two-tailed Student’s t test was used to calculate the significance of these means compared to controls with Prism software (GraphPad Software, Inc.).

**Immunofluorescent staining.** Freshly harvested tissue was immediately frozen in OCT embedding medium and stored at -80°C until use. Five-micron-thick frozen sections were fixed in cold 4% paraformaldehyde for 5 min, then stained with primary antibodies rat anti-mouse CD31 monoclonal antibody (1:100 dilution; BD Biosciences), rabbit anti-mouse phospho-Akt (Ser473, 1:50 dilution; Upstate Biotechnology), or rabbit anti-mouse phospho-S6 ribosomal protein polyclonal antibody (1:200 dilution; Cell Signaling Technology) overnight at 4°C. Tissues were then incubated in appropriate FITC- or Cy3-conjugated secondary antibody (1:100 dilution; Jackson ImmunoResearch) for 1 h at room temperature. Images were captured using a Nikon TE2000 inverted microscope equipped with differential interference contrast microscopic/phase/fluorescence optics, connected to a Leica DC200 digital camera and analyzed using DCViewer software.

**WST-1 assay.** WST-1 assay for cell viability was done according to the directions of the manufacturer (Roche Applied Science). Briefly, subconfluent cells in 96-well plates were treated with rapamycin (1–100 ng/mL) in minimal media supplemented with 2% FCS for 72 h, at which time 10 μL of WST-1 reagent was added to each well in a final volume of 100 μL per well. Cells were incubated for 4 h, after which the absorbance of samples was measured against a background control as blank at 450 nm.

**Western blot analysis.** Subconfluent cells in 10-cm plates were treated with rapamycin (1–50 ng/mL) in minimal media supplemented with 2% FCS for 48 h. Cells were harvested and cell lysates were analyzed by Western blot according to standard protocols. Blots were probed with antibodies to phosphorylated Akt (pAkt; Ser473), phosphorylated S6 kinase (pS6K; Ser244/246), total S6 kinase (total S6 kinase; Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology, Inc.) with reverse transcription. In addition, dilutions of the cDNA were run to ensure the efficiency of the reactions. Statistical significance was achieved in all sets both for triplicates within experiments and repeats of experiments. The statistical analysis was done on the iCT (dCT) values, and treatment groups in which these values were not significantly different are marked “n.s.” on the graph that shows fold changes in expression. The formula used to calculate fold change was FC = 2^{-（dCT）}, with dCT calculated as the difference in CT values between VE-cadherin and GAPDH. Primers used for quantitative reverse transcription-PCR of VE-cadherin were sense, 5' ggcctggcaagctga 3' and antisense, 5' tctggagagagctc 3’. Primers used for GAPDH were sense, 5' ggcattcaacggcacg 3' and antisense, 5' aagatgtagccaggctc 3'.

**Vascular content.** A quantitative assessment of tumor vascular content was measured by taking one third to one half of individual tumors and measuring the relative amount of VE-cadherin mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the total RNA fraction using quantitative real-time reverse transcription-PCR as described (12). Triplicates were run and required to be within 0.1 SE to be used for further analysis. Controls included samples ± Taq Polymerase, and RNA alone without reverse transcription. In addition, dilutions of the cDNA were run to ensure the efficiency of the reactions. Statistical significance was achieved in all sets both for triplicates within experiments and repeats of experiments. The statistical analysis was done on the iCT (dCT) values, and treatment groups in which these values were not significantly different are marked “n.s.” on the graph that shows fold changes in expression. The formula used to calculate fold change was FC = 2^{-（dCT）}, with dCT calculated as the difference in CT values between VE-cadherin and GAPDH. Primers used for quantitative reverse transcription-PCR of VE-cadherin were sense, 5' ggcctggcaagctga 3' and antisense, 5' tctggagagagctc 3’. Primers used for GAPDH were sense, 5' ggcattcaacggcacg 3' and antisense, 5' aagatgtagccaggctc 3'.
Results and Discussion

Prolonged treatment of cells with rapamycin, as in a therapeutic setting, inhibits signaling downstream of mTOR, which can have either positive or negative feedback effects on the upstream Akt pathway (9, 13, 14). One mechanism that has been proposed for rapamycin inhibition of Akt activation is the inhibition of mTOR-rictor complex 2 assembly, a kinase for Akt at Ser473 (13, 15). Alternatively, increased pAkt signaling has been attributed to the release of feedback inhibition from S6 kinase to insulin-responsive substrate-1, which promotes pAkt signaling (14). We have treated MCF7 breast cancer cells and stromal cells in vitro for 48 h with rapamycin in a dose range from 1 to 50 ng/mL. Reproducibly, we observed a bimodal pAkt response in primary human endothelial cells and primary human dermal fibroblasts with increased pAkt levels at 1 ng/mL of rapamycin followed by a dose-dependent decrease in pAkt from 4 to 50 ng/mL of rapamycin (Fig. 1A). Similar dose-dependent responses to rapamycin were also observed in primary human mammary fibroblasts. However, rapamycin had a different effect in MCF7 cells. There was a dose-dependent increase in pAkt levels in response to rapamycin, which remained elevated at rapamycin concentrations up to 50 ng/mL of rapamycin followed by a dose-dependent increase in pAkt levels (Fig. 1A). Similar treatment of another tumor cell line, HeLa cells, with rapamycin showed that the decrease in pAkt levels occurred at much higher drug concentrations than in stromal cells (data not shown). In all cell types tested, pS6 kinase was effectively inhibited at much lower drug doses than pAkt, although in tumor cells, pS6 kinase was still more refractory to rapamycin than primary endothelial cells and fibroblasts (Fig. 1A). These data, in combination with those published by others (14, 16), suggest that tumor cells are more resistant to rapamycin inhibition of pAkt than stromal cells. We next tested cell viability in response to rapamycin and found that in general, the relative viability of these cells reflected their pAkt response (Fig. 1A). In human dermal endothelial cells and human dermal fibroblasts, cell viability was reduced; however, in MCF7 cells, there was an increase in cell viability at lower drug concentrations, which plateaued at higher drug concentrations.

In order to determine the pharmacologically relevant doses of rapamycin for treatment in a mouse model of breast carcinoma, we performed a rapamycin dose-response study in mice of the same FVB genetic background as our tumor model. We observed that the rapamycin blood levels recommended for clinical use in transplant patients for immunosuppression (6–15 ng/mL; ref. 17) were achieved between 0.1 and 0.5 mg/kg/d after 8 days of treatment in mice (Fig. 2A). Using the mouse spontaneous mammary tumor model MMTV-PyT, we tested the ability of rapamycin to inhibit tumor growth at these doses. We initiated rapamycin treatment when the tumors first became palpable, at which point they had passed the angiogenic switch and were 50 to 100 mm³. We observed effective inhibition of tumor growth in control DTG mice at 0.1 and 0.5 mg/kg/d (Fig. 2B). We have previously shown that these doses of rapamycin were effective at reducing pAkt levels in the tumor vasculature, suggesting that endothelial Akt signaling may be an important target of the antitumor efficacy of rapamycin (9). Consistent with these findings, dosing of rapamycin in a mouse model of colon cancer was important for drug efficacy, and continuous infusions of low drug doses was more effective at reducing tumor growth than bolus dosing (18). Rapamycin blood levels were 15 ng/mL under those conditions. Metronomic dosing of chemotherapeutic drugs have been found to be most effective for targeting the stroma (19). Clinical trials of rapamycin in breast cancer have reported the use of a dosing schedule similar to standard chemotherapy and significantly different from the daily dosing schedule for rapamycin in

Figure 2. Rapamycin blood levels in FVB mice and MMTV-PyT tumor growth in response to rapamycin.

A. trough blood levels of rapamycin in FVB mice after 8 d of treatment, i.p. injections (n = 5 animals per group). B. growth of MMTV-PyT tumors in DTG mice treated with 0, 0.1, and 0.5 mg/kg/d of rapamycin for 1 mo initiated after tumors became palpable. Points, means; bars, SD. Both 0.1 and 0.5 mg/kg rapamycin treatment groups had means that were significantly different from untreated controls (P < 0.002).
transplant patients. The standard chemotherapy dosing may be less effective than our metronomic dosing if indeed the real tumor target is the stroma. Rapamycin has been shown to reduce tumor cell proliferation and increase apoptosis in breast tumors (20). In addition, our previous work showed that rapamycin decreases Akt signaling and vascular permeability in tumor endothelium (9).

Therefore, to test whether the antitumor response to rapamycin was limited by endothelial Akt signaling, we did a similar course of drug treatment in TTG animals that were littermates of the control DTG animals shown in Fig. 2B. In addition to forming spontaneous mammary tumors, TTG mice also had endothelial cell–specific expression of myrAkt that was repressible by tetracycline. To assess the response to rapamycin in animals with elevated endothelial Akt signaling, tetracycline was removed when tumors first became palpable. Removal of tetracycline permitted the expression of endothelial myrAkt and rapamycin treatment was initiated at this time. Among the untreated groups, the overall tumor growth in TTG mice was similar to that in DTG mice, indicating that endothelial Akt activation did not confer a growth advantage to tumors per se (Fig. 3A). However, TTG tumors with constitutive endothelial Akt activation showed increased resistance to rapamycin treatment at both low and high drug doses (Fig. 3B). We have used a particular VE-cadherin/tTA transgenic line (D4 line) that when crossed with TET/myrAkt mice, exhibited a modest increase in myrAkt expression and a slow alteration in the systemic vasculature, causing it to resemble the tumor vasculature. The systemic vascular leak and edema lead to death of the animals after 6 to 7 weeks of myrAkt induction (9). In the course of this study, the animals remained overtly healthy, although a nonstatistically significant trend for reduced tumor growth in the untreated TTG began after 15 days of myrAkt expression compared with control DTG animals (Fig. 3A). Our interpretation is that this late-trend drop in tumor growth was due to the decreased systemic vascular function that we previously reported rather than directly due to Akt expression in the tumor vasculature (9).

We predicted that reduction in vascular permeability in tumors would lead to reduction in interstitial fluid pressure, which usually is reflected by more “open” vessel lumens. Visualization of the tumor vasculature with anti-CD31 antibody to label endothelial cells in tumor tissue sections revealed that rapamycin treatment reduced the number of tumor vessels but also resulted in more opened blood vessels (Fig. 3C). We have sampled tumor sections from viable portions of both smaller and larger tumor nodules, thus representing a range of tumor sizes, and did not observe open lumens in untreated tumors. Even though the vessel morphology differed with rapamycin treatment, there were no striking

Figure 3. Effects of increased endothelial Akt activation on MMTV-PyT tumor response to rapamycin. A, time course of tumor growth in untreated DTG and TTG mice. Points, means; bars, SD. B, time course of tumor growth in DTG and TTG mice treated with 0.1 and 0.5 mg/kg of rapamycin. DTG and TTG mice were matched sets of littermates. Differences in mean tumor volume for DTG versus TTG were statistically significant for each rapamycin dose (P < 0.001). C, representative tumor sections stained with anti-CD31 antibody (green) to highlight the tumor vasculature. Untreated tumors and tumors treated with 0.5 mg/kg/d of rapamycin (magnification, ×200). D, RNA was isolated from tumors at the end of the study and quantified for endogenous VE-cadherin mRNA levels by real-time reverse transcription-PCR as a read-out of endothelial cell content. The average fold change in treated DTG and TTG tumors relative to corresponding untreated tumors. Data for 0.1 and 0.5 mg/kg rapamycin for each genotype were combined into one group. Combining the treatment groups did not alter the relative responses or statistical significance. n.s., not statistically significant; n, number of tumors analyzed.
differences in pericyte or mural cell coverage between untreated and treated tumors as determined by smooth muscle actin staining of tumor sections (data not shown).

To quantify the vascular content in large areas of tumor, we used a quantitative method to provide an average relative vascular content. Real-time quantitative reverse transcription-PCR analysis for endothelial cell–specific VE-cadherin mRNA levels in whole tumor lysates allowed us to obtain a relative value for the vascular content in control and treated tumor samples (Fig. 3D). This methodology has been useful in measuring vascular content in several contexts including developing organs (12). We observed that rapamycin reduced the vascular content in DTG tumors. Consistent with the decreased rapamycin efficacy of tumor growth inhibition in TTG animals, we did not observe significantly reduced vascular content from tumor to tumor in the untreated TTG tumors, consistent with the increased variation seen in tumor growth in the time period that these RNA samples were obtained (see Fig. 3A, days 15–20). Overall, the data from control animals were consistent with other reports that rapamycin was antiangiogenic and reduced vascular density (18, 21). Because we observed a difference between stromal cells and tumor cells in pAkt response to rapamycin in vitro (Fig. 1), we next assessed the levels of pAkt in MMTV-PyT tumors by Western blot analysis of whole tumor lysates, in which the majority of the cells were tumor cells (Fig. 4A). Quantitation of Western blots of multiple tumor lysates (n = 3 animals per group) by densitometry showed that statistically significant increases in pAkt levels were observed in both DTG and TTG tumors treated with 0.5 mg/kg of rapamycin as compared with corresponding untreated tumors or tumors treated with a lower dose of rapamycin (0.1 mg/kg; Fig. 4A and B). Both DTG and TTG total tumor lysates had reduced pS6 kinase levels at 0.5 mg/kg of rapamycin as compared with corresponding untreated tumors or tumors treated with a low dose of rapamycin (0.1 mg/kg; Fig. 4A and C). However, the inhibitory effects of rapamycin at 0.5 mg/kg on S6 kinase phosphorylation was less in TTG than in DTG animals, suggesting that increased endothelial Akt activation in the vasculature led to increased resistance to rapamycin downstream of mTOR (Fig. 4C).

We failed to observe any significant changes in either pAkt or pS6 kinase levels in total lysates from tumors treated with 0.1 mg/kg of rapamycin. However, even at this low drug dose (corresponding to trough blood levels of 5.47 ng/mL, which is at the low end of the therapeutic range in patients), significant inhibition of tumor growth was observed (Figs. 2B and 3A).

To differentiate tumor cell and endothelial cell pAkt levels in response to rapamycin, tumor sections were double-stained for...
CD31 (to label endothelial cells) and pAkt, or CD31 and phosphorylated S6 ribosomal protein (pS6), another marker of Akt/mTOR activation (Fig. 4D). In untreated TTG animals, there was abundant pAkt in both tumor cells and blood vessels. However, in rapamycin-treated animals, pAkt levels in tumors blood vessels were significantly reduced, whereas pAkt levels in tumor cells were not significantly altered. Similar findings in tumor sections were seen with pS6. Thus, even in the setting of sustained endothelial Akt activation in TTG tumors, rapamycin effectively blocked Akt/mTOR signaling in tumor endothelial cells. Our in vitro studies showed that rapamycin, even at low concentrations, inhibited pAkt and pS6 kinase as well as cell viability in human endothelial cells and fibroblasts (Fig. 1A). Taken together, these findings suggest that the stromal response to rapamycin was more sensitive than the tumor response and is important for tumor growth inhibition.

As we have previously shown, sustained Akt activation significantly increases vascular permeability (9). We showed that rapamycin reduced tumor vascular permeability, which could lead to reduced interstitial fluid pressure and better perfusion of the tumor, thus providing more effective delivery of the drug and improved antitumor effects. Our observations of more open vessel lumens in rapamycin-treated tumors are consistent with our previous finding of reduced tumor vascular permeability. It has been difficult to determine whether the characteristic alterations in tumor vascular structure and function provide a benefit to the tumor. Our data supports the hypothesis that reducing vascular abnormalities correlated with reduced tumor growth in the TTG rapamycin cohort. Further supporting this hypothesis are the findings that reduction of vascular hyperpermeability by endothelial nitric oxide synthase inhibitors delays tumor progression in mice without direct cytostatic or antiangiogenic effects (22). Thus, we propose that clinical anticancer treatment with rapamycin is antiangiogenic in nature, and may be beneficial even at low doses at which the antitumor effects reflect vascular normalization rather than frank reduction in vascular density. A combined benefit might be expected in combination therapy of standard chemotherapy plus rapamycin because improved vascular function has been proposed to increase the efficacy of drug distribution within tumors (23). Two studies have reported a synergy between rapamycin and doxorubicin in a myc-driven mouse model of lymphoma (24), and between rapamycin and paclitaxel in breast cancer xenografts models (25). These two studies focused on tumor cell apoptosis with the assumption that the findings were due to direct effects on tumor cells, but did not address optimal drug schedules and dosing, or whether there was a vascular component to their synergy.

Our observations that rapamycin is similarly effective at reducing Akt activation and cell viability of other stromal components, such as fibroblasts, may suggest that rapamycin is acting as an overall stromal inhibitor rather than strictly as an angiogenic inhibitor. A test of the importance of rapamycin-mediated stromal inhibition awaits model systems to induce Akt activation in other stromal cells besides endothelial cells. Although we cannot exclude the possibility that rapamycin has direct inhibitory effects on tumor cell proliferation and metabolic pathways via inhibition of mTOR and its downstream effectors, our data do suggest that the endothelial Akt response plays a significant role in the antitumor efficacy of rapamycin. The findings in our in vitro and in vivo studies highlight the dose dependence of rapamycin activity. This is consistent with previous studies that report optimal efficacy at low doses and with continuous dosing (18, 21, 26). Thus, careful consideration of rapamycin dosing and scheduling is indicated for optimal use of rapamycin in cancer treatment.

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References


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Correction: Rapamycin Is an Endothelial Akt Inhibitor

In the article on how rapamycin is an endothelial Akt inhibitor in the June 1, 2007 issue of Cancer Research (1), there is an error in Fig. 3B. The corrected figure appears below.

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