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ORIGINAL ARTICLE

Dosing of rapamycin is critical to achieve an optimal antiangiogenic effect against cancer

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Summary

Rapamycin has antiangiogenic activity against tumors. This has been discussed while addressing the problem of cancer in organ transplantation. Here we investigated effective dosing schedules against tumors and angiogenesis. Growth of established CT-26 colon adenocarcinoma tumors was measured in Balb/c mice treated with total equivalent rapamycin doses (1.5 mg/kg/day) given once a day, once every 3 days, or by continuous infusion. Tumors were most inhibited with continuous rapamycin infusion, and less by bolus dosing. Interestingly, however, continuous dosing produced the lowest rapamycin blood levels (15 ng/ml). As rapamycin-sensitive p70S6-kinase intracellular signaling is critical for angiogenesis, p70S6-kinase activation was measured in endothelial cells by Western blotting. Maximal p70S6-kinase inhibition occurred from 1–5 ng/ml rapamycin. These same rapamycin concentrations optimally blocked vessel-sprouting from cultured aortic rings. Therefore, low-level rapamycin dosing most effectively controls tumors in mice. Importantly, antiangiogenic rapamycin levels are compatible with immunosuppressive doses, supporting its potential use in transplant patients with cancer.

Introduction

Development of cancer is an increasingly common complication of long-term immunosuppressive therapy in transplant patients [1]. A growing concern for the problem is at least partially rooted in the accumulating number of transplant patients on long-term immunosuppression, many of which are just now entering a post-transplant period where the risk for cancer becomes high. To address this problem, therapeutic protocols need to be developed to simultaneously attack emerging or established malignancies, while protecting an allograft from immunologic destruction. In this respect, we have recently shown that the immunosuppressive drug rapamycin has potent effects on tumor growth [2]. Importantly, one of the mechanisms for this anticancer effect is based on the antiangiogenic properties of the drug, related to blockage of vascular endothelial growth factor (VEGF) production and stimulation of endothelial cells. Angiogenesis, which refers to the growth of new blood vessels, occurs primarily during embryonic development, and in adults, is triggered locally in finely balanced processes such as wound healing [3]. However, angiogenesis is reactivated in a wide range of human diseases in response to pathological stimuli. In particular, tumor growth is dependent on angiogenesis, making this process a new and exciting target to control cancer [4]. The goal of the present study was to determine an optimal dosing schedule for the immunosuppressive-antiangiogenic drug rapamycin, with regard to its anticancer potential in mice. Data from these studies show that optimal antiangiogenic effects with rapamycin in mice are achieved by maintaining relatively low doses of drug on a constant basis. Importantly, the most effective doses are compatible with those used for immunosupppression treatment in organ transplantation.

Materials and methods

Mice and tumor model

Male 20–25 g Balb/c mice (Harlan Winkelmann, Borchen, Germany) were used and housed under standard conditions. Prior to all surgical procedures, animals were anaesthetized with ketamine hydrochloride and xylazine, according to animal procedures approved by the regional authorities.

CT-26 cells used in our experiments were derived from a murine Balb/c colon adenocarcinoma [5]. Tumor cells were maintained by standard cell culture techniques prior to injection into a subcutaneous location. To produce established tumors in mice, 1×10^6 tumor cells were injected subcutaneously in the dorsal region of syngenic Balb/c mice, as described previously [2]. In all experiments tumors were allowed to grow for 7 days without any treatment. At this time, rapamycin treatment was initiated for the remainder of the experimental period under three different dosing schedules that delivered the same total amount of drug over the test period: (i) 1.5 mg/kg/day, intraperitoneally (i.p.), (ii) 4.5 mg/kg once every 3 days i.p., or (iii) 1.5 mg/kg/day by continuous infusion using an i.p. placed Alzet pump (model 2002; DURECT Corporation, Cupertino, CA, USA). For Alzet pumps, rapamycin was dissolved in dimethylsulfoxide (DMSO). Control mice received DMSO alone in osmotic pumps for the continuous delivery experiments; to control bolus i.p. injections, a group of mice received saline. Tumor volumes were estimated by measurements of the short and long axis of the mass, where $V = \pi/6 \times a^2 \times b$ (a is the short axis of the tumor and b, the long axis). Animals were monitored for tumor size, as well as for their general condition. Tumor-bearing mice showing signs of inactivity, cachexia, or poor responsiveness were killed. For comparison purposes, whole blood was collected in some mice and levels of rapamycin were determined either 2 h after bolus i.p. drug application, or 24 h after the last dosing (testing kindly provided by Prof. Kaever at the Medizinische Hochschule Hannover, Institute for Pharmacology, Hannover, Germany). Samples were taken from these mice only after the treatment had been initiated for at least 7 days. Notably, the baseline total daily dose of 1.5 mg/kg/day was selected for experiments in the present study because this amount is generally necessary to prolong allograft survival in mice, and we have previously shown this dose to be effective against tumor growth [2].

Aortic ring assay

Aortic ring assays were performed using a modification of the technique reported by Nicosia [6]. Briefly, thoracic aortae were harvested from 6–8-week-old male Lewis rats (Harlan Winkelmann). After rinsing with ice-cold saline to remove blood clots, and dissection of adherent connective tissue, the aorta was sectioned into 1-mm slices that were placed immediately on matrigel-coated 24-well plates. HEPES-buffered DMEM medium containing rapamycin at different concentrations was added to cultures, and the plate was incubated at 37 °C, 5% CO_2, for 4 days. Fresh medium with respective additives was re-introduced into the cultures on day 2. At the end of the incubation period phase-contrast photomicrographs of the rings were taken to record the formation of vessel sprouts. We also measured the sprouting area around the ring using Image-J computer software (US National Institutes of Health).

Human umbilical vein endothelial cell culture and p70S6-kinase Western blotting

Human umbilical vein endothelial cell (HUVEC) cultures were purchased from PromoCell (Heidelberg, Germany) and were maintained in Falcon 'surface-modified', polystyrene flasks with growth factor supplemented ('Supplement Pack'; PromoCell) endothelial-cell basal medium (PromoCell) containing 2% fetal bovine serum, as detailed by the manufacturer. Human versus mouse endothelial cells were selected for our study to better understand which concentrations of rapamycin might be effective against angiogenesis in clinical organ transplantation.

For Western blotting analysis of p70S6-kinase phosphorylation at the Thr^{389} site (known site of molecule activation [7]), HUVEC were cultured in fully supplemented medium, or were first starved ('diet' medium: serum and supplement depleted) for 10 h, and then treated with diet medium containing increasing concentrations of rapamycin for 1 h. After this conditioning period, HUVEC were stimulated with 50 ng/ml recombinant $VEGF_{165}$ (R&D Systems, Wiesbaden, Germany) for 20 min, and then cell extracts were prepared with sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein extract were separated on polyacrylamide SDS gels, transferred, and probed with rabbit anti-phospho (Thr^{389}) p70S6-kinase antibody (New England Labs, Frankfurt, Germany). Detection of the primary antibody was performed with a secondary goat anti-rabbit horseradish peroxidase antibody (DAKO A/S, Glostrup, Denmark), using the ECL Western blotting system (Amersham, Freiburg, Germany). To compare protein-gel loading, β-actin was detected in the same way in samples with a primary goat antibody against β-actin, followed by a donkey anti-goat horseradish peroxidase antibody (antibodies from Santa Cruz, Santa Cruz, CA, USA).

Statistical analysis

Data are given as mean ± SEM in quantitative experiments. For statistical analysis of differences between the groups, a Student's *t*-test was performed.

Results

Balb/c mice bearing an established subcutaneous CT-26 colon adenocarcinoma were treated with a total dose of 1.5 mg/kg/day, delivered i.p. by daily injection, by intermittent 3-day bolus injection, or by a continuous pumping system. Results from these experiments show that control mice receiving either saline or DMSO pump carrier solution died or were sacrificed in <3 weeks after treatment as a result of tumor growth (Fig. 1). Mice receiving bolus 1.5 mg/kg/day injections of rapamycin generally survived with tumor growth inhibition, and tumor volume stabilized by the end of the treatment period. In comparison, those mice receiving a higher bolus rapamycin dose every 3 days did not exhibit more inhibition of tumor growth, and in fact, tumors tended to grow at a higher rate than with the daily dosing schedule. Considering that mice with larger tumors dropped out of the 4.5 mg/kg/3 day treatment group by day 28 (Fig. 1), mean tumor size is slightly underestimated at this point, but still more than the once-a-day bolus injection group. The most effective inhibition of tumor growth occurred in rapamycin-treated mice on the continuous pumping system. Tumor growth with the continuous delivery strategy was significantly less than bolus injections starting on day 11, and remained lower during the entire 35-day experimental period. Survival data also show the most positive effect with continuous drug delivery. Measurement of rapamycin blood levels with each regimen revealed that trough levels in the daily bolus-dosed mice were just under 40 ng/ml, while the continuous pumping system yielded levels only near 15 ng/ml (Table 1). As could be expected, mice receiving the high bolus dose of rapamycin every 3 days showed greater drug blood levels at 2 and 24 h postinjection, in comparison with the other delivery schedules. Therefore, attaining high peak concentrations of rapamycin by bolus dosing did not improve the anti-tumor response, and in fact, low continuous dosing of drug provided the most effective treatment schedule.

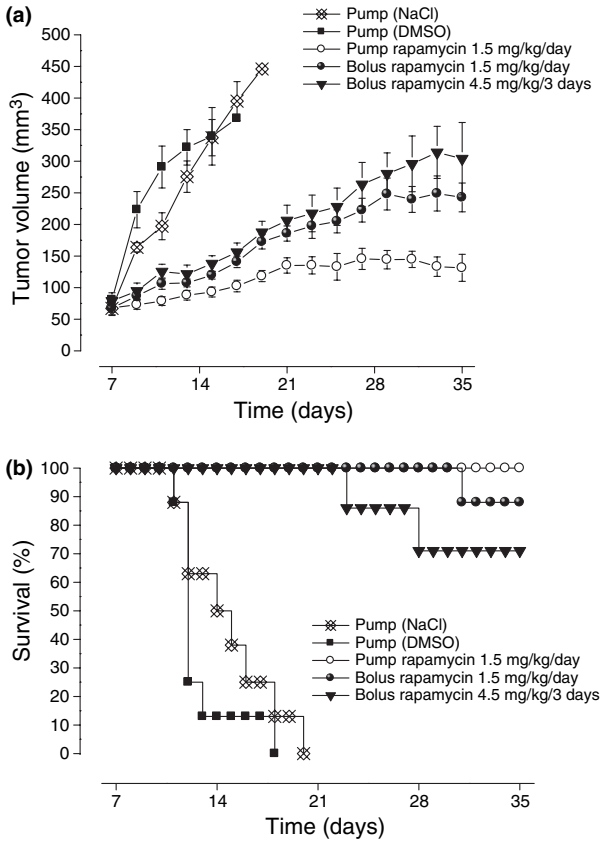

Figure 1 Effect of different rapamycin delivery schedules on CT-26 colon adenocarcinoma growth in Balb/c mice. Subcutaneous CT-26 tumors were established for 7 days before beginning treatment with rapamycin. Treatment regimens consisted of rapamycin given i.p. at 1.5 mg/kg/day ($n = 8$), 4.5 mg/kg once every 3 days ($n = 8$), or 1.5 mg/kg/day by a continuous infusion pump ($n = 7$). Controls for i.p. injections were given saline each day ($n = 8$), or for pump controls, pumps with DMSO carrier solution were placed in mice ($n = 8$). (a) The tumor volume (mean ± SEM) measured every other day after treatment; (b) animal survival in each of the test groups. There was a significant difference in tumor volume in continuous infusion mice after day 11, compared with either of the two bolus injection groups ($P \leq 0.03$).

To more specifically test the rapamycin dose needed to produce an optimal antiangiogenic effect, we performed *in vitro* experiments using an aortic ring–vessel sprouting assay. With this system, intact sections of rat aorta are cultured on a matrigel matrix, and sprouting directly from aortic tissue is measured. Data from these experiments show that rapamycin began to inhibit sprouting from aortic rings at 0.1 ng/ml, and near complete inhibition occurred at concentrations of 1 ng/ml or greater

Table 1. Rapamycin blood levels after different treatment schedules.

Group	24-h level* (ng/ml)	2-h level† (ng/ml)
1.5 mg/kg/day bolus	39 ± 7	488 ± 190
1.5 mg/kg/day pump	15 ± 1	–
4.5 mg/kg/3 days bolus	72 ± 39	841 ± 81

All samples were obtained from mice at least 7 days after initiation of therapy.
*24 h after the last bolus i.p. injection ($n = 4$ each).
†2 h after the last bolus i.p. injection ($n = 4$ each).

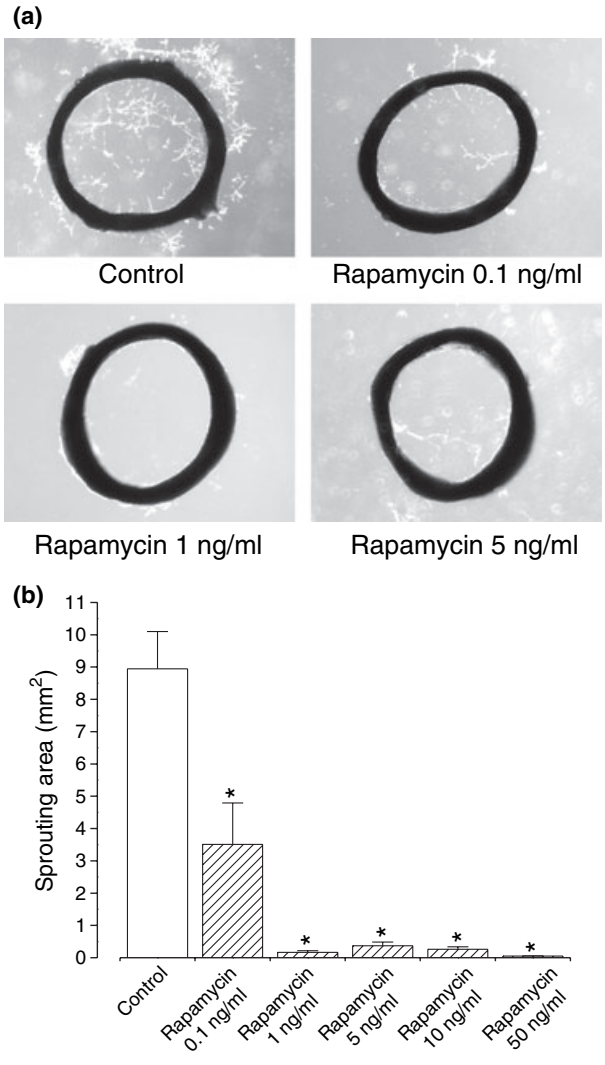

Figure 2 Dose–response effect of rapamycin on *in vitro* angiogenesis. Different rapamycin doses were tested in an assay measuring vascular-sprouting from cultured rat aortic rings. (a) Photomicrographs of aortic rings 4 days after culture with increasing concentrations of rapamycin. Rapamycin blocked vessel sprouting at concentrations ≥1 ng/ml. (b) Quantitative computer-assisted analysis of the sprouting area around the aortic rings. Results from the complete range of rapamycin concentrations tested is shown (0.1–50 ng/ml); $*P < 0.05$, versus control. Values are expressed as the mean ± SEM obtained from four separate experiments.

(Fig. 2), indicating a very high sensitivity of vascular-sprouting to low drug concentrations.

To better understand the sensitivity of angiogenesis to rapamycin, we investigated the activity of a key intracellular signaling molecule necessary for endothelial cell stimulation and proliferation (and IL-2-mediated lymphocyte stimulation [8]), p70S6-kinase. Rapamycin inhibits this signaling pathway because mTOR, which is blocked by rapamycin, is an upstream regulator of p70S6-kinase.

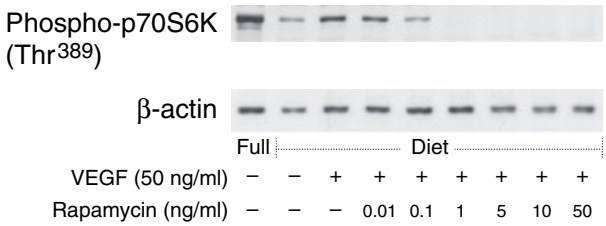

Figure 3 Effect of rapamycin on p70S6-kinase activation. HUVEC were cultured with increasing concentrations of rapamycin, and VEGF-induced phosphorylation of p70S6-kinase at the Thr^{389} site was measured by Western blotting. 'Full' stimulation refers to culturing of HUVEC with medium containing fetal bovine serum and supplemental growth factors. 'Diet' conditions refer to culturing without either fetal bovine serum or supplements. In the experiment, one control HUVEC culture was maintained under 'full' culture conditions, and the remainder were put under 'diet' conditions for 10 h, then rapamycin was added for 1 h, as indicated. To induce p70S6-kinase phosphorylation in the presence or absence of rapamycin, VEGF (50 ng/ml) was added to selected cultures for 20 min, and all cells were subsequently harvested for Western blotting. Blots were probed with an antibody against phospho(Thr^{389} site) p70S6-kinase, and loading of protein was controlled with a β-actin-specific antibody.

Here we tested the activation of p70S6-kinase by measuring its level of phosphorylation at the Thr^{389} site in the presence of increasing concentrations of rapamycin. Phosphorylation at the Thr^{389} site correlates with p70S6-kinase activity [7]. Under full stimulation culture conditions, including the presence of serum and supplemental growth factors, p70S6-kinase phosphorylation was increased in HUVEC, compared with serum and supplement-starved cells (Fig. 3). Addition of recombinant VEGF, which is known to stimulate endothelial cells through this signaling pathway [9], also stimulated the activation-phosphorylation of p70S6-kinase. Importantly, VEGF-mediated stimulation of HUVEC p70S6-kinase phosphorylation could be partially inhibited at a rapamycin concentration of 0.1 ng/ml, with complete inhibition occurring at 1 and 5 ng/ml. Therefore, understanding that VEGF plays a critical role in angiogenesis, these results also suggest that only low levels of rapamycin are needed to have a potent effect on tumor blood vessel formation.

Discussion

Therapeutic strategies aimed at treating and preventing cancer in organ transplant patients are needed to improve long-term allograft and patient survival. The problem faced in immunosuppressed patients with cancer is that removal or lowering of immunosuppression can help control tumor growth, but the transplanted organ is then placed at an increased risk for rejection. This dilemma has recently motivated researchers to seek immunosuppressive agents that do not enhance tumor formation or

growth. Recent studies indeed suggest that not all immunosuppressive agents promote cancer, and some even possess properties that lead to tumor inhibition. One of the most promising agents in this respect is rapamycin, which not only acts in some respects as an anti-metastatic [10] and antiproliferative agent [11,12], it can deprive tumors of vital nutrients via a potent antiangiogenic effect [2]. Results from the present study show that proper administration of rapamycin is an essential consideration to obtain an optimal anti-tumor effect, and importantly, that doses of rapamycin used in transplantation for immunosuppression overlap with those needed for its most potent antiangiogenic activity.

Data from our study clearly show that steady-state rapamycin doses in the low ng/ml range have an optimal inhibitory effect on angiogenesis and growth of established tumors in mice. Bolus injections of rapamycin did increase drug (peak and trough) levels in treated mice versus continuous infusion, but higher drug levels did not serve to increase the inhibitory effect on tumor growth. In fact, our results indicate that *in vitro* angiogenesis from aortic rings could be maximally blocked with rapamycin at concentrations as low as 1 ng/ml. Consistent with this finding, when we examined the activation status of the p70S6-kinase intracellular signaling pathway, which is known to be inhibited by rapamycin and is required for VEGF signaling to endothelial cells, complete blockage of p70S6-kinase phosphorylation also occurred at ≥1 ng/ml of drug. Therefore, our results suggest that to attain an optimal antiangiogenic effect against occurring tumors, high doses of rapamycin are not likely to provide any beneficial effect. Indeed, in a previous study [2] we have shown that increasing the dose of rapamycin (4.5 mg/kg/day) can have a slight beneficial effect on tumors early on, but after some time tumors re-entered a phase of rapid growth, leading to a high animal morbidity and mortality. Our present experiments using equivalent total dosages of rapamycin suggest a similar effect because large bolus doses of drug produced the poorest reduction in tumor growth and early animal loss because of tumor effects and possibly toxicity. The explanation for this phenomenon is not fully understood, however, it can be speculated that higher concentrations of rapamycin have numerous side-effects leading to deterioration in general health condition [13], giving cancer cells a growth advantage that allows unabated tumor progression. In addition, it is possible that severe immunosuppression induced by high drug concentrations may give the cancer an immunologically based growth advantage. Notwithstanding the low-dose effect we observe, it must also be considered that rapamycin does have cytotoxic effects on various tumor cells, but that the concentrations of drug necessary for these effects tend to be in the μg or mg/ml range [11]. Indeed, direct rapamycin effects on tumor cell cytotoxicity or proliferation will probably vary widely from one tumor type to another, and with some cancers, may even be quite potent (in the low ng/ml range) when the neoplastic cell shows a specific dependency on the phosphatidylinositol 3-kinase – p70S6-kinase intracellular signaling pathway [14–16]. With mouse CT-26 adenocarcinoma and B16 melanoma cells tested in our own laboratory, an appreciable sensitivity to rapamycin has only been observed at concentrations of approximately 1 μg/ml [2]. Taken together, we suggest that the effectiveness of rapamycin as an anticancer agent falls into two primary dose ranges, where low ng/ml concentrations of drug produce a potent antiangiogenic or potential antiproliferative effect, and higher doses which can serve to have a direct cytotoxic effect on tumor cells (Fig. 4). Importantly, with regard to the use of the drug in transplant patients, the antiangiogenic dosing profile of rapamycin appears most compatible with normal immunosuppressive therapy.

Finally, it is interesting to consider the observation that continuous delivery of rapamycin provided the most effective *in vivo* treatment regimen. This finding is similar to what has been observed with other known antiangiogenic agents, where low-level continuous-delivery methods induce the greatest anti-tumor effect [17–19]. The advantage of this type of therapy is that side effects tend to be low, making it possible to use this approach over a long period of time to maintain constant 'pressure' against tumor expansion. It is notable that as antiangiogenic effects are directed against normal vessel cells, and not cancer cells, drug resistance to this form of therapy is less likely [4]. However, an antiangiogenic approach to tumor therapy is not without pitfalls. In particular, if the

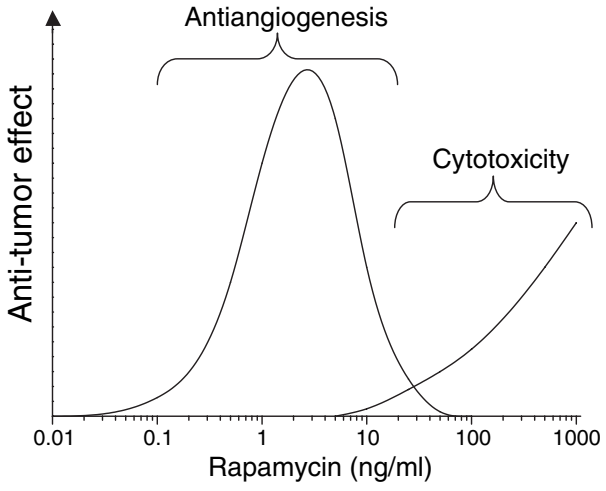

Figure 4 Theoretical representation of expected antiangiogenic and cytotoxic effects of rapamycin treatment on tumors.

antiangiogenic agent is not also cytotoxic at the concentrations used, nests of cancer cells not large enough to require angiogenesis will continue to exist [20]. Therefore, if a cure is the primary goal of therapy, it is likely that rapamycin will need to be combined with a cytotoxic agent capable of directly killing residual cancer cells as they emerge from a dormant state. It would be logical to speculate that control of cancer with a combined approach might require long-term antiangiogenic treatment with intermittent cytotoxic therapy. Our challenge in organ transplantation over the next years will be to design safe, scientifically sound, protocols that can help to treat and prevent cancer in this high-risk population, with a primary goal of maximizing allograft and patient survival.

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