Inhibition of mTOR Radiosensitizes Soft Tissue Sarcoma and Tumor Vasculature

James D. Murphy, Aaron C. Spalding, Yash R. Somnay, Sonja Markwart, Michael E. Ray, and Daniel A. Hamstra

Abstract

Purpose: The PI3K/Akt/mTOR prosurvival pathway is frequently up-regulated in soft tissue sarcoma. Mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin, have recently shown clinical benefit in soft tissue sarcoma, and mTOR inhibition has also been associated with radiosensitization of carcinoma and endothelial cells. This study tested the hypothesis that rapamycin radiosensitizes soft tissue sarcoma and endothelial cells in vitro and in vivo through the inhibition of mTOR.

Experimental Design: Colony formation assays were done to determine the radiosensitizing properties of rapamycin on three human soft tissue sarcoma cell lines (SK-LMS-1, SW-872, and HT-1080) and human dermal microvascular endothelial cells (HDMEC). The functional effects of rapamycin and radiation on the endothelial compartment were evaluated with microvascular sprouting assays. The in vivo radiosensitizing activity of rapamycin was assessed with s.c. SK-LMS-1 nude mice xenografts treated with concurrent daily rapamycin, radiation, or both for three weeks.

Results: In vitro radiosensitization was shown in all three soft tissue sarcoma cell lines with minimally cytotoxic doses of rapamycin. SK-LMS-1 xenografts displayed significant tumor growth delay with rapamycin and radiation compared with either treatment alone. Radiation resulted in transient increased mTOR function, whereas rapamycin abolished this signaling in irradiated and unirradiated samples. In HDMEC, rapamycin and radiation reduced microvessel sprouting, but did not alter colony formation.

Conclusions: Minimally cytotoxic concentrations of rapamycin inhibited the mTOR cascade in culture and in vivo while radiosensitizing soft tissue sarcoma, and produced synergistic effects with radiation on HDMEC microvessel formation. By targeting both tumor and endothelial compartments, rapamycin produced potent radiosensitization of soft tissue sarcoma xenografts. Clinical trials combining rapamycin and radiotherapy in soft tissue sarcoma are warranted.
Translational Relevance

The treatment of soft tissue sarcoma is often a challenge requiring multimodality therapy. Recently, molecularly targeted inhibitors have been investigated as treatment options in oncology. In particular, agents that target tumor vasculature are now beginning to be used in a variety of cancers. Rapamycin is a well-tolerated drug used extensively in organ transplant patients because of its antirejection properties. In addition, rapamycin, by down-regulating the mammalian target of rapamycin (mTOR) signaling pathway, has shown single-agent activity in soft tissue sarcoma. In this study, we show that clinically achievable concentrations of rapamycin sensitize both soft tissue sarcoma cells and tumor vasculature to the effects of ionizing radiation. In vivo this combination therapy resulted in greater than additive inhibition of tumor vascular growth and tumor growth delay. These results suggest that combined mTOR inhibition with radiation may provide a unique opportunity to increase tumor control in soft tissue sarcomas.

mTOR inhibitors have been shown to inhibit angiogenesis (20, 21, 29) and to sensitize tumor vasculature to ionizing radiation (30). The mechanism of in vivo radiosensitization has not been fully elucidated, but these findings suggest that combining mTOR inhibition with radiation results in radiosensitization of both the primary tumor and the cells within the vascular endothelial compartment.

Sarcomas, which can originate from any anatomic site (31), are a rare and heterogeneous group of cancers thought to arise from mesenchymal tissues (32). In sites such as the extremity, surgical resection with radiotherapy have resulted in local control rates surpassing 90% (33, 34). However, in sites such as the retroperitoneum and deep trunk, attaining complete control rates surpassing 90% (33, 34). However, in sites such as the above. For angiogenesis evaluation, a capillary sprouting assay was performed. For angiogenesis evaluation, a capillary sprouting assay was performed.

Colony formation assay. Radiosensitization was assessed after a 90-min exposure to rapamycin with radiation delivered 60 min into rapamycin treatment. Controls were treated with the vehicle alone, and all conditions received equal amounts of ethanol, PEG400, and Tween80. Upon completion of treatment, floating cells were collected and added to the trypsinized adherent cells. This combination of cells was then washed with PBS, counted on a Coulter Counter, and replated in media with 10% fetal bovine serum at clonal density. After 5 to 10 d of incubation, colonies were fixed with 7:1 methanol:acetic acid, and stained with 0.5% crystal violet. Only colonies with >50 cells were counted. Each condition per colony forming assay was plated in triplicate. Each complete colony forming assay was repeated at least once, with representative data shown.

Endothelial cell culture and sprouting assay. Human dermal microvascular endothelial cells (HDMEC; Cell systems) were used to investigate the effects of rapamycin on endothelial cell growth and function. HDMEC were maintained in EGM-2 media (Lonza) supplemented with 50 ng/mL of rhVEGF 165. One hour prior to radiation the cells were changed to fresh media plus the appropriate concentration of rapamycin. Immediately after irradiation the cells were transferred to ice, washed, and prepared for Western analysis as noted above. For angio genesis evaluation, a capillary sprouting assay was done (39). Six-well plates were precoated with collagen (Cohesion). HDMECs were added to each well, and allowed to adhere overnight. Cells were treated with 50 ng/mL rhVEGF 165 in fresh media daily. The day after plating, cells were treated with vehicle or rapamycin for 48 h, and received radiation after 24 h into rapamycin treatment. The number of tubular structures were assessed daily with a phase microscope at 200×, counting six random high-powered fields per well, with three wells per condition. The fractional product method was utilized to test for evidence of synergy (40).

Tumor growth experiments. Exponentially growing SK-LMS-1 cells (5 × 103) were suspended in 200 μL PBS and injected s.c. into the mice. The effect of rapamycin on inhibition of tumor growth, the time to tumor size doubling, and the mean tumor volume at 24 d post-treatment was assessed. The tumor volume was determined using the equation: Tumor volume at 24 d post-treatment was assessed. The tumor volume was determined using the equation:
flanks of 6- to 8-week-old athymic male nude mice (Charles River Labs). Treatment commenced once tumors reached an average volume of 150 mm³. Mice were randomized into four treatment groups (control, rapamycin alone, radiation alone, and rapamycin plus radiation) with seven mice per arm. Based on an \textit{a priori} power analyses using a similar dataset (41), it was determined that seven mice would provide adequate power (81\%) to detect a 1.5-fold difference in growth delay. Treatment commenced on day 1, and rapamycin was administered via i.p. injection on days 1-5, 8-12, and 15-19, at a dose of 2 mg/kg. Radiation was given daily in 2 Gy fractions, 1 h after rapamycin injection, for a total dose of 30 Gy. Mice were anesthetized with i.p. injections of ketamine (80 mg/kg) and xylazine (4 mg/kg), and were placed prone in custom-made holders. Cerrobend shielding was placed over the entire mouse minus the tumor creating a conformal treatment field. After radiation, the mice were kept warm with heating pads until fully recovered from anesthesia. Animals were handled

**Fig. 1.** mTOR inhibition sensitizes soft tissue sarcoma to radiation. SK-LMS-1 cells treated with increasing doses of rapamycin for 60 min and prepared for Western blot (A) and densitometric analysis (B). SK-LMS-1 cells treated with rapamycin for 60 min, irradiated, harvested immediately, and prepared for Western blot (C) and densitometric analysis (D). Data points (B and D), mean ± SE. Colony forming assay as described in Materials and Methods for SK-LMS-1 cells (E), HT-1080 cells (F), and SW-872 cells (G). Data points (E, F, and G), average ± SE of triplicate plating from one colony forming assay.
Table 1. Cytotoxicity with rapamycin in soft tissue sarcoma

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<tr>
<th>Cell line</th>
<th>Surviving fraction</th>
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<tr>
<td></td>
<td>3 nmol/L</td>
</tr>
<tr>
<td>SK-LMS-1</td>
<td>99% ± 4%</td>
</tr>
<tr>
<td>HT-1080</td>
<td>76% ± 6%</td>
</tr>
<tr>
<td>SW-872</td>
<td>66% ± 3%</td>
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According to the University of Michigan Laboratory Animals Maintenance Manual.

Tumor volumes \(V\) were estimated using the volume of an ellipsoid: \(V = \frac{k}{6}D_1 \times D_2^2 \), where \(D_1\) and \(D_2\) are the longer and shorter perpendicular diameters, respectively. Tumor growth was expressed as relative change in volume compared with day 1. Growth delay (GD) of a treatment arm (GD_{rap+RT}) was defined as the time interval from day 1 through when a tumor reached 5 times its day 1 volume. The growth delay enhancement ratio (GDER) was defined as the following: \(GDER = \frac{\text{GD}_{\text{rap+RT} \& - \text{minus}}}{\text{GD}_{\text{rap alone}} / \text{GD}_{\text{RT alone}}}\). GDER > 1 implies synergism between rapamycin and radiation. Animals were euthanized when tumor volumes reached 1.5 cm\(^3\). Additionally, one animal from each group was sacrificed on day 5 of treatment, 30 min after receiving radiation and the tumor was prepared for immunohistochemistry.

Immunohistochemistry and tumor vasculature analysis. The harvested xenograft tumors described above were formalin-fixed, paraffin-embedded, and stained with an anti-CD31 antibody. Microvascular density was quantified by counting the number of blood vessels per 400 \(\times\) field. Six random fields per tumor were selected, and the results indicate the average ± SE of the six counts.

Results

mTOR signaling is increased with radiation and inhibited with rapamycin. In order to assess the response of soft tissue sarcoma cell lines to rapamycin alone, SK-LMS-1 cells were exposed to increasing doses of rapamycin for one hour prior to analysis. Treatment with rapamycin decreased phosphorylation of mTOR in a dose-dependent fashion, and completely abolished phosphorylation of p70s6k, which is directly downstream of mTOR and is a common marker of mTOR function (Fig. 1A and B), whereas levels of total mTOR and total p70s6k remained unchanged.

Irradiation of SK-LMS-1 cells produced a transient increase in the amount of phosphorylated Akt, but the increase in phosphorylated mTOR was less pronounced. Accordingly, we observed a transient increase in phospho-p70s6k, whereas the expression of total p70s6k remained unchanged. This radiation-induced increase in phospho-p70s6k was inhibited by treatment with rapamycin (Fig. 1C and D). Stimulation with fetal bovine serum (10%) was used as a positive control as it is known to increase signaling down the mTOR pathway, and this too was inhibited with rapamycin treatment.

Rapamycin sensitizes soft tissue sarcoma to radiation in vitro. Having ascertained that rapamycin treatment could inhibit mTOR signaling in soft tissue sarcoma, we next assessed if this would lead to radiosensitization. We first established the cytotoxicity profile of rapamycin alone prior to examining its potential as a radiosensitizer. After a 90-minute treatment with doses up to 300 nmol/L rapamycin, the surviving fraction of SK-LMS-1 cells was not significantly decreased as assessed by colony formation assay. However, this same dose of rapamycin was moderately toxic to the HT-1080 and SW-872 cell lines (Tables 1). Nontoxic to moderately cytotoxic (IC\(_{50}\)) doses were chosen to evaluate the radiosensitizing properties of rapamycin. All three soft tissue sarcoma cell lines showed radiosensitization to rapamycin (Fig. 1E, F, and G), with DERs...
in the range of 1.2 to 1.3. Additionally, the observed radiosensitization was dose-dependent, with maximal sensitivity to radiation occurring with more cytotoxic doses of rapamycin.

**Rapamycin and radiation cause decreased vascular sprouting.** To determine the effects of rapamycin and radiation on vascular endothelial cells, monolayers of HDMEC in culture were treated with rapamycin or radiation. As with soft tissue sarcoma cells, one-hour incubation with rapamycin even at doses as low as 3 nmol/L was sufficient to inhibit phospho-p70s6k without changing the expression of total p70s6k (Fig. 2A and B). Unlike with soft tissue sarcoma cells, however, radiation did not increase the phosphorylation of p70s6k. Furthermore, unlike with soft tissue sarcoma which were radiosensitized with noncytotoxic concentrations of rapamycin that inhibited p70s6k phosphorylation, we were unable to radiosensitize HDMECs with conventional colony formation assay even at doses of drug that abolished p70s6k phosphorylation (Fig. 2C).

With the use of a vascular sprouting assay, HDMECs were treated with rapamycin (3 nmol/L), radiation (2 Gy), or the combination, and microvessel sprout formation was then monitored to assess HDMEC function in addition to cytotoxicity. Qualitatively, the combination of clinically relevant doses of rapamycin and radiation resulted in a dramatic inhibition of microvessel formation that was greater than either treatment alone (Supplemental Fig. S1), despite the fact that this combination did not result in any increase in cytotoxicity to HDMEC. Quantitatively, radiation alone caused a modest decrease in microvessel sprout formation by 21 ± 6%, and 3 nmol/L rapamycin similarly caused a 34 ± 12% inhibition (Fig. 2D). More importantly, the combination of radiation and rapamycin resulted in 73 ± 5% inhibition of sprout formation (P < 0.01 by one-way ANOVA). The observed inhibition of microvessel formation in HDMEC treated with rapamycin and radiation was greater than would be expected by a simple additive effect (P < 0.05, Supplemental Table S1). This suggests synergy between rapamycin and radiation, resulting in greater reduction of endothelial sprouting than with either treatment alone.

**Rapamycin is a potent radiosensitizer in vivo.** Having ascertained that clinically achievable doses of rapamycin and radiation could potentially sensitize both the tumor and vascular compartments in vitro, we next assessed the combination of rapamycin and radiotherapy in vivo using SK-LMS-1 xenografts (Table 2). A treatment schedule consisting of daily rapamycin and daily fractionated radiation was chosen to mimic a regimen that could be used in a clinical setting. Initial evaluation determined that daily i.p. rapamycin doses of 2 mg/kg produced a slight growth delay in comparison with control tumors (data not shown). Additionally, a single dose of 2 mg/kg rapamycin inhibited mTOR activity as shown by reduction of phospho-p70s6k in both irradiated and unirradiated tumors (Fig. 3A) while not altering total p70s6k expression.

Using this combination regimen, xenografts treated with daily rapamycin and radiation resulted in significant tumor GD when compared with either modality alone (Fig. 3B). The GD for tumors treated with single modality radiation or rapamycin were 5.7 ± 3.7 days and 10.1 ± 1.2 days, respectively, whereas the GD for tumors treated with both modalities was 35.9 ± 2.6 days (P < 0.0001 compared with radiation or rapamycin alone). The GD enhancement ratio for this combined rapamycin and radiation group was 4.6, indicating significant synergism with rapamycin and radiation. Treatment-related toxicity measured as percent body weight loss was minimal, with the greatest weight loss occurring in the rapamycin plus radiation group. Weight loss in this group was <5%, and all animals had returned to their starting weight within 3 days of the end of treatment, suggesting that this treatment regimen was well tolerated.

**mTOR inhibition and radiation reduce microvessel density in vivo.** To investigate the in vivo effects of rapamycin on tumor vasculature, we harvested tumors on day 5 of treatment, and quantified microvessel density as based upon staining for CD31, a marker for vascular endothelial cells (Fig. 4). After just 5 days of treatment, tumors treated with radiation (Fig. 4B) or rapamycin (Fig. 4C) showed 38 ± 6% and 38 ± 6% reductions, respectively, in microvessel formation when compared with controls (Fig. 4A; P < 0.005 for each). Confirming the effect

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<th>Cell line</th>
<th>Dose enhancement ratio</th>
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<td></td>
<td>3 nmol/L</td>
</tr>
<tr>
<td>SK-LMS-1</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>HT-1080</td>
<td>1.1 ± 0.04</td>
</tr>
<tr>
<td>SW-872</td>
<td>1.04 ± 0.22</td>
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**Table 2. In vitro sarcoma radiosensitization with rapamycin.**
observed in vitro, the combination of rapamycin and radiation (Fig. 4D) resulted in a 75 ± 5% reduction when compared with control-treated tumors (P < 0.0005). In addition, the combination treatment was more effective in reducing the number of CD31-positive cells than either radiation or rapamycin treatment alone (P < 0.0005).

**Discussion**

The mTOR pathway is frequently up-regulated in a variety of sarcoma histologies. A recent study examining patient tumor samples found that between 78% and 90% of leiomyosarcomas, malignant fibrous histiocytomas, and dedifferentiated liposarcomas have increased phosphorylated Akt (3). This study also found significantly increased levels of phospho-p70s6k and eIF-4BP, particularly in leiomyosarcomas and malignant fibrous histiocytoma histologies. Another report retrospectively examined rhabdomyosarcoma tumor samples and found that increased phospho-Akt, phospho-p70s6k, and phospho-4EBP1 were associated with poor recurrence-free and overall survival (42). A clinical trial with the Akt inhibitor perifosine has shown prolonged responses in the subset of patients with advanced soft tissue sarcoma (43). Another promising large phase II clinical trial with the rapamycin analogue AP23573 showed a 27% clinical benefit response in soft tissue sarcoma patients (24). These clinical and histologic studies provide compelling evidence of sarcoma’s dependence on the mTOR pathway.

The key finding of this present study is that inhibition of mTOR with rapamycin sensitizes both sarcoma cells and tumor vasculature to radiation, and targeting both tumor and endothelial compartments produced potent radiosensitization in soft tissue sarcoma xenografts. This is the first study to show the radiosensitizing effects of mTOR inhibition with both in vitro and in vivo preclinical models of sarcoma. Prior research suggested that in vitro radiosensitization secondary to mTOR inhibition is cell line–specific, and likely dependent upon dysregulation of the PI3K/Akt/mTOR pathway. For example, breast cancer and PTEN-deficient prostate cancer cell lines showed radiosensitization with mTOR inhibition as a monolayer in culture (25, 27). It has also been postulated that this in vitro sensitivity to mTOR inhibition may depend upon the feedback loop between Akt and mTOR such that mTOR inhibition would only be expected to result in radiosensitization in the presence of deregulated Akt (44). In contrast, U87 and GL261 glioma cells were not radiosensitized by mTOR inhibition in vitro, but both cell lines showed increased sensitivity to radiotherapy with mTOR inhibitors in xenograft models, suggesting that in vitro models may not adequately address the unique interaction among radiation, mTOR inhibition, and the tumor microenvironment (28, 30). Finally, the degree of sensitization in xenografts may be schedule-dependent, for in a second study using U87 xenografts this combination was only additive (45). Interestingly, all of these studies utilized hypofractionated radiation schedules with doses of radiation on the order of 4 to 5 Gy delivered over 5 to 7 fractions. It is possible that these studies took advantage of the postulated increased vascular effects of ionizing radiation by using larger than conventional radiation fractions (46).

The supra-additive effects of mTOR inhibition and radiation shown in vivo are likely in part secondary to radiosensitization of the vascular compartment. Previously, it was found that rapamycin sensitized human umbilical vein cells to radiation in vitro, but this study utilized significantly higher doses of rapamycin (100 nmol/L) than would be clinically achievable in humans (30). Using a clinically relevant dose of rapamycin (3 nmol/L ref. 47), we did not appreciate radiosensitization with HDMECs via colony formation assays. However, we found

![Fig. 4. Rapamycin and radiotherapy decrease tumor microvessel density. Mice bearing SK-LMS-1 xenografted tumors were treated with rapamycin (2 mg/kg) and radiation (2 Gy) daily for 5 d. Tumors were harvested, fixed, and stained with anti-CD31 antibody to show tumor microvasculature. Images represent (A) control, (B) rapamycin alone, (C) radiation alone, and (D) both rapamycin and radiation.](image)
that rapamycin and radiation caused markedly decreased vascular sprout formation even in the absence of cytotoxicity likely due to down-regulation of angiogenic cytokines such as vascular endothelial growth factor (21, 39, 48). Although our xenograft experiments utilized doses of rapamycin that are higher than achievable in human serum, Luker et al. (49), using a novel bioluminescent system, found that in vivo inhibition of the interaction between mTOR and FK506 binding protein has a Kᵢ of only 1.5 ± 0.3 nmol/L, which they confirmed in vitro and which would be clinically achievable and would concur with the inhibition of the phosphorylation of p70s6k that we observed in HDMEC with low levels of rapamycin. Taken together, these findings suggest that the antiangiogenic effects of rapamycin and radiotherapy may be effective in treating a variety of malignancies regardless if the tumor cells themselves show significant radiosensitization to rapamycin.

The precise mechanism of cell death induced by the combination of radiation and mTOR inhibition is unclear, but others have implicated both apoptosis (25) and autophagy (50). Prior studies have also linked irradiation with a transient increase in signaling down the Akt/mTOR survival pathway, paradoxically associating radiotherapy with activating radio-resistant tumor cells (29). Thus, rapamycin and rapamycin analogues may inhibit paradoxically associating radiotherapy with activating radio-sensitivity of tumor cells themselves shows significant radiosensitization to rapamycin.

In conclusion, mTOR inhibition radiosensitized both the tumor and vascular compartments, and produced potent in vivo radiosensitization. In light of the recent encouraging clinical data with mTOR inhibitors as monotherapy, our study provides compelling preclinical evidence for translating this combination into clinical practice in the treatment of soft tissue sarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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