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Inhibition of the Mammalian Target of Rapamycin Sensitizes U87 Xenografts to Fractionated Radiation Therapy[1]

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ABSTRACT

The mammalian target of rapamycin (mTOR) modulates key signaling pathways that promote uncontrolled proliferation of glioblastoma multiforme (GBM). Because rapid tumor proliferation may contribute to the clinical radioresistance of GBM tumors, the combination of rapamycin, a selective mTOR inhibitor, and radiation was studied *in vitro* and *in vivo* in a GBM model. In monolayer cultures of U87 and SKMG-3 cells, rapamycin had no impact on radiation sensitivity. In contrast, rapamycin significantly enhanced the efficacy of fractionated radiation of established U87 xenografts in nude mice. Similar effects were seen in U87 spheroids treated with rapamycin and radiation, which suggests that the sensitizing effects of this drug are dependent on disruption of mTOR signaling pathways specifically within tumor cells. Inhibition of these signaling pathways can lead to inhibition of G_1-specific cyclin-dependent kinase activities, and this could contribute to the sensitizing effects of rapamycin. Consistent with this idea, roscovitine, a specific cyclin-dependent kinase inhibitor, also enhanced the efficacy of fractionated radiation in U87 spheroids. These data demonstrate that inhibition of tumor proliferation does not diminish the efficacy of fractionated radiation and suggest that disruption of key signal transduction pathways may significantly enhance the effectiveness of radiation therapy in malignant gliomas.

INTRODUCTION

GBM[3] is the most common primary central nervous system tumor in adults and is uniformly fatal despite aggressive therapy. Intensive research over the past three decades has focused on combining different cytotoxic chemotherapies with radiation in an effort to improve survival. Unfortunately, these approaches have had no impact on treatment outcome, and the standard of care continues to be surgical resection followed by external beam radiation therapy. GBMs are clinically radioresistant, and the majority of tumors recur within or at the margin of the radiation field. Biological factors in GBM that may contribute to this clinical radioresistance include intrinsic cellular radioresistance, rapid proliferative rate, invasiveness, and tumor hypoxia (1–6). With an increasing understanding of the cellular and molecular mechanisms governing these processes, combining radiation therapy with novel therapeutic agents specifically targeting one or more of these factors holds promise for improving the outcome of therapy in this difficult disease.

The mTOR is a key signaling molecule in GBM that drives uncontrolled tumor proliferation. mTOR is a serine-threonine kinase that functions downstream from Akt in a phosphatidylinositol 3′-kinase/Akt/mTOR signaling pathway. This pathway is commonly activated in GBM through constitutive activation of upstream receptor tyrosine kinases, such as epidermal growth factor receptor, and/or loss of PTEN tumor suppressor function (7–11). In response to mitogenic stimuli, mTOR regulates the phosphorylation of p70 S6 kinase and eIF4E-binding protein 1, which promotes translation of select mRNA transcripts (reviewed in Ref. 12). Selective inhibition of mTOR by the macrolide antibiotic rapamycin blocks phosphorylation of these two regulatory proteins and leads to cell cycle arrest through up-regulation of $p27^{kip1}$, a CDK inhibitor, and down-regulation of cyclin D1 (13–15). Rapamycin inhibits mTOR signaling at low nanomolar concentrations only when it is bound in a complex with the endogenous FK506-binding protein FKBP-12. The interaction of the FKBP12-rapamycin complex with mTOR is highly specific, and therefore cellular and biochemical effects of rapamycin are generally believed to result exclusively from inhibition of mTOR signaling (16, 17).

Rapamycin is well tolerated in patients and is a Food and Drug Administration-approved immunosuppressant for the prevention of solid organ transplant rejection (18). Screening by the National Cancer Institute revealed that rapamycin potently inhibited cell proliferation in a number of tumor types including prostate, breast, and glioblastoma cell lines. Rapamycin also inhibited tumor growth in animals, and these observations prompted the development of two rapamycin analogues, CCI-779 and RAD001, which currently are being evaluated in early clinical trials as anticancer agents. Anecdotal experience with CCI-779 in these trials suggests promising activity in several tumor types including malignant gliomas.

Proliferation of tumor cells during a 6- or 7-week course of radiation therapy can repopulate a tumor and decrease the efficacy of radiation treatment (19, 20). This suggests that pharmacological inhibition of tumor repopulation with a cytostatic agent might enhance the overall efficacy of fractionated radiation (21). However, growth-arrested cells held in confluence can be more radioresistant than actively cycling cells (22), which suggests that cytostatic agents might actually increase the radiation resistance of tumors. To test these potentially conflicting hypotheses, the effects of rapamycin on radiation response were evaluated both *in vitro* and *in vivo*. In monolayer culture, rapamycin inhibited proliferation of U87 and SKMG-3 malignant glioma cell lines without any significant change in radioresistance. In contrast, rapamycin treatment significantly enhanced the efficacy of fractionated radiation in U87 flank xenografts. Consistent with inhibition of repopulation, rapamycin treatment decreased tumor proliferation in these xenografts. Moreover, similar "sensitization" was observed in U87 spheroids treated *in vitro* with fractionated radiation in the presence of either rapamycin or the selective cell cycle inhibitor roscovitine. These studies suggest that novel cell cycle inhibitors might be used in combination with fractionated radiation therapy to inhibit tumor repopulation and improve local tumor control.

MATERIALS AND METHODS

Cell Culture and Antibodies. A172, U87, and U118 malignant glioma cell lines were maintained in DMEM (Life Technologies, Inc.), and SKMG-3 cells were maintained in α-MEM (BioWhitaker), respectively. Both media were supplemented with 10% fetal bovine serum and 10 mM HEPES. Cell lines were obtained from Dr. C. David James. Rapamycin (NSC 226080) was obtained

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[3] The abbreviations used are: GBM, glioblastoma multiforme; mTOR, mammalian target of rapamycin; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; BrdUrd, bromodeoxyuridine; CI, confidence interval; CDK, cyclin-dependent kinase; VEGF, vascular endothelial growth factor.

from the National Cancer Institute Developmental Therapeutics Program[4] and dissolved in ethanol to yield a 5 mg/ml stock solution, which was stored at −20°C. The drug was diluted in media immediately before treatment of cells. Antibodies specific for p70 S6 kinase were obtained from Santa Cruz Biotechnology (sc-230) and Cell Signaling (catalogue number 9202). An antibody that specifically recognized phosphorylation of p70 S6 kinase on Thr-389 was obtained from Cell Signaling (catalogue number 9205).

Immunoprecipitation and Western Blotting. Cells cultured in 100-mm tissue culture dishes were harvested for assays during exponential growth. The cells were washed twice with PBS and then scraped on ice in 1 ml of lysis buffer [25 mM Tris, 50 mM NaCl, 10% glycerol, and 1% Triton X-100 (pH 7.4) containing 50 mM β-glycerol phosphate, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 10 μg/ml leupeptin, and 20 nM microcystin].

Lysates were cleared of insoluble material by centrifugation, and equivalent amounts of protein (1 mg) were incubated on ice for 30 min with p70 S6 kinase-specific antibodies (Santa Cruz Biotechnology). The immune complexes were precipitated with protein A-Sepharose beads, and the resulting immunoprecipitates were washed twice in lysis buffer. Samples were boiled in 1× SDS sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were probed with 1 μg/ml phospho-specific Thr-389 antibody diluted in Tris-buffered saline containing 0.02% Tween 20 and 5% nonfat dried milk. After washing in Tris-buffered saline containing 0.02% Tween 20, membranes were incubated with a secondary polyclonal rabbit antimouse IgG antibody conjugated to horseradish peroxidase (Cell Signaling). Membranes were developed with Super Signal Chemiluminescence reagent (Pierce). Finally, the blots were stripped and reprobed with non-phospho-specific p70 S6 kinase antibodies (Cell Signaling).

Clonogenic Assay. The effect of rapamycin on the radiosensitivity of U87 and SKMG-3 cells was assessed in a clonogenic assay. Cells were treated with 0 or 100 nM rapamycin diluted in media for 24 h before trypsinization and resuspension in fresh growth medium. Portions of the cells were processed for cell cycle analysis, whereas the remaining cells were treated in a clonogenic assay. Cells were irradiated with a ^{137}Cs source at a dose rate of 6.4 Gy/min in suspension culture and immediately plated in triplicate 60-mm dishes at cell concentrations estimated to yield 20–100 colonies/dish. To maximize plating efficiency, up to 50,000 lethally irradiated U87 feeder cells were added to the U87 plates (plating efficiency, 9%). No feeder cells were required for the SKMG-3 cells (plating efficiency, 34%). The final concentration of ethanol, used as the drug solvent, did not exceed 0.1% (v/v), and this solvent concentration had no effect on either the clonogenicity or radiosensitivity of either cell line (data not shown). Cells were cultured for 2 weeks before fixation and staining with Coomassie Blue. Colonies with >50 cells were scored.

MTS Assay. Cells in exponential growth were harvested and plated in 96-well plates (1500 cells/well in 80 μl of standard growth medium). Each treatment condition was tested in six replicate wells. Cells were incubated overnight, and then graded concentrations of rapamycin were added to the wells in 20 μl of media. Cells were incubated at 37°C for 72 h and then processed for the MTS assay (Promega) according to the manufacturer's instructions. After incubation of cells with the MTS reagent for 2 h, absorbance at 490 nm was measured in a spectrophotometer.

Cell Cycle Analysis. Cells were fixed in 70% ethanol diluted in PBS, and the samples were stored at −20°C. The fixed cells were resuspended in PBS containing 20 μg/ml propidium iodide and 100 μg/ml boiled RNase A and incubated for 30 min at 37°C before flow cytometric analysis on a Becton Dickinson FACScan. Twenty-thousand ungated events were collected. Cell cycle distribution was determined using the ModFit software package (Verity) after excluding doublets and clumps by gating on the DNA pulse-width *versus* pulse-area displays. To measure BrdUrd incorporation, animals received an i.p. injection of 1 mg of BrdUrd 30 min before euthanasia. Tumors were removed from the animals, diced into small pieces, and fixed in 70% ethanol diluted in PBS. Samples were processed for flow cytometry as described previously and analyzed on a Becton Dickinson FACScan (23). After excluding clumps and doublets, a bivariate distribution of green height (BrdUrd-FITC) and red area (cell cycle/propidium iodide) was analyzed to quantitate the fraction of BrdUrd-positive nuclei.

[4] http://dtp.nci.nih.gov/docs/misc/available_samples/dtp_indsamples.html.

Regrowth Delay Assay. Flank xenografts were established in 8–10-week-old female athymic nude mice by s.c. injection of 2–5 million U87 cells resuspended in 50 μl of media. Approximately 4 weeks after injection, animals with established xenografts were stratified by size and randomized into four treatment groups: (*a*) control; (*b*) radiation only; (*c*) rapamycin only; and (*d*) rapamycin + radiation. For irradiation, unanesthetized animals were immobilized in a lead jig that shielded the head, thorax, and upper abdomen. Radiation was delivered at a dose rate of 4 Gy/min through a single posterior to anterior 300 kVp unfiltered photon beam (half-value layer, 2.73 mm Al). For rapamycin injections, stock rapamycin was diluted first in sterile 10% PEG400/8% ethanol and then in an equal volume of sterile 10% Tween 80 for a final concentration of 20 μg rapamycin/100 μl. Rapamycin was delivered by i.p. injection, and the doses of rapamycin were calculated assuming that all mice weighed 20 g. Tumors were measured with calipers in three dimensions, 3–5 times/week. Tumor volume was calculated using the formula for volume of an ellipsoid: $4/3\pi \times L/2 \times W/2 \times H/2$, where L = length, W = width, and H = height. Time for tumor regrowth to three times the initial volume was calculated for each animal. Regrowth delay was calculated as the difference in the mean regrowth times for any pair of treatments.

Spheroid Culture. U87 cells were initially plated on Petri dishes to induce spheroid formation and subsequently transferred to spinner flasks. Cultures were stirred continuously for 4–7 days. For the regrowth delay experiments, single spheroids were transferred to individual wells of a multiwell plate that had been previously coated with 1% agarose dissolved in DMEM. The indicated drug concentrations were added 1 h before irradiation. Spheroids were irradiated in air at room temperature as described above for the radiation clonogenic assays. Spheroids were measured in two dimensions 3×/week using an optical micrometer in an inverted light microscope. The volume of a spheroid was estimated using the formula $4/3\pi \times L/2 \times W/2 \times W/2$, where the width, W, is the shorter of the two dimensions. The time for spheroid regrowth to 10 times initial volume was calculated for each spheroid. Regrowth delay is calculated as the difference in the mean regrowth times for any pair of treatments.

Statistics. A two-tailed Student's t test was used to establish statistical significance between control and rapamycin treatment for the MTS and flow cytometry data. Results from two independent animal regrowth delay experiments were pooled for statistical analysis. CIs for the regrowth delay were calculated based on a pooled estimator of variance (s_p^2) using the following formula: $CI = t \times s_p \times (1/n_1 + 1/n_2)^{-2}$, where t is obtained from a t-distribution with $(n_1 + n_2 - 2)$ degrees of freedom. Data from the spheroid regrowth delay assays were handled in a similar manner. Data from two independent determinations of BrdUrd labeling index were pooled, and Wilcoxon's rank-sum test was used to test the difference between rapamycin and control treatment.

RESULTS

Rapamycin Inhibits Cell Proliferation *in Vitro*. The effects of rapamycin on proliferation were examined in four glioma cell lines using a MTS assay. This assay relies on the bioreduction of a tetrazolium compound by metabolically active cells into a soluble formazan salt, which then can be quantitated using a spectrophotometer. As seen in Fig. 1*A*, incubation with 100 nM rapamycin for 72 h significantly inhibited the proliferation of A172, SKMG-3, U87, and U118 cells. Based on these data, we elected to study the effects of rapamycin on SKMG-3 and U87 cells in more detail. The observed decrease in proliferation after treatment with 100 nM rapamycin corresponded with a significant accumulation of cells in the G_0-G_1 compartment ($P < 0.001$), as early as 24 h after drug addition, and a corresponding decrease in the fraction of cells traversing S phase ($P < 0.001$; Fig. 1*B*). Consistent with a noncytotoxic mechanism of action, rapamycin did not induce apoptosis or diminish clonogenic cell survival (data not shown).

Rapamycin Does Not Affect Radiation Sensitivity *in Vitro*. Classic radiobiological studies have demonstrated that noncycling cells can be relatively radioresistant (22, 24). This suggests that combining a cytostatic agent with radiation might actually increase the radiation resistance of tumor cells. To address this concern di-

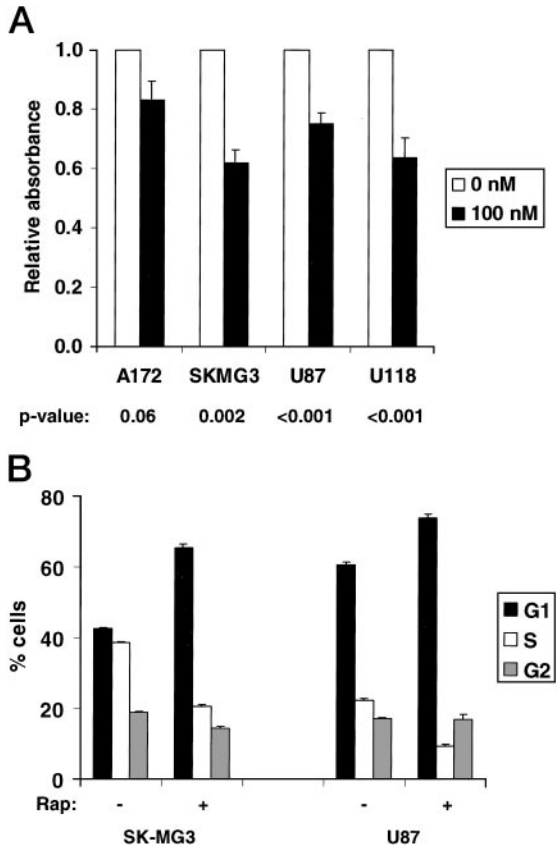

Fig. 1. Rapamycin inhibits proliferation. *A*, A172, SKMG-3, U87, and U118 cells were incubated with 0 or 100 nM rapamycin for 72 h and then processed in a MTS assay. In this assay, absorbance at a wavelength of 490 nm is proportional to cell number. The absorbance for drug-treated cells was normalized to that of control-treated cells, and the results shown represent the mean ± SE from four independent experiments. *B*, cells were incubated with 0 or 100 nM rapamycin for 24 h, and the cell cycle distribution was determined by flow cytometric analysis. Results shown represent the mean ± SE of triplicate samples from a single experiment. Identical results were obtained in three independent experiments. The effects of rapamycin on the distribution of cells in G_0-G_1 and S phase were statistically significantly for both cell lines ($P < 0.001$).

rectly, U87 and SKMG-3 cells were incubated with 100 nM rapamycin for 24 h and then simultaneously processed for flow cytometric analysis and radiation clonogenic survival assays. As shown in Figs. 2, *A* and *B*, preincubation with rapamycin had no effect on radiation survival of U87 and SKMG-3 cells, despite accumulation of cells in G_0-G_1 and a corresponding decrease in the S-phase fraction. The results in SKMG-3 cells are especially noteworthy in that rapamycin treatment caused a marked G_0-G_1 arrest and yet had absolutely no effect on radiation sensitivity compared with control-treated cells. Similar results were obtained with U251, A172, and U118 malignant glioma cell lines, and incubation of cells with rapamycin after irradiation also had no effect on radiosensitivity (data not shown). Taken together, these data indicate that rapamycin-induced cell cycle arrest does not increase the radioresistance of glioma cell lines growing in monolayer culture.

Rapamycin-mediated Inhibition of mTOR Signaling. The planned combination studies with rapamycin and radiation required identification of a rapamycin dosing regimen that inhibits mTOR signaling in xenografts. Because SKMG-3 cells are not tumorigenic in nude mice, these experiments were performed in U87 cells. Previous studies suggest that the rapamycin-FKBP12-mTOR complex is extremely stable and that rapamycin is essentially an irreversible inhibitor of mTOR (25). Consistent with these results, incubation of U87 cells with 10 nM rapamycin for 1 h completely inhibited mTOR-dependent phosphorylation of p70 S6 kinase (Fig. 3*A*), and mTOR signaling remained completely inhibited for at least 72 h after replacement of the drug-containing media with fresh media. Using a similar approach, the dose of rapamycin required to inhibit mTOR signaling in established U87 xenografts was identified. The i.p. injection of either 0.1 or 1 mg/kg rapamycin resulted in complete inhibition of p70 S6 kinase phosphorylation 24 h after drug treatment (Fig. 3*B*). These data suggest that adequate inhibition of mTOR signaling would be achieved by intermittent dosing with 1 mg/kg rapamycin delivered by i.p. injection once every 3 days.

Rapamycin Enhances the Efficacy of Radiation in U87 Xenografts. Proliferation of tumor cell clonogens between radiation doses can significantly affect the overall efficacy of therapy (19, 20). Rapamycin inhibits proliferation of U87 cells, and the rapamycin-induced cell cycle arrest does not increase the radioresistance of these cells. Therefore, we hypothesized that the combination of rapamycin with fractionated radiation therapy should improve the efficacy of treatment of U87 tumors. This hypothesis was tested in a tumor regrowth delay assay using U87 flank xenografts grown in nude mice. Because tumor proliferation between radiation fractions was of specific interest, radiation was given in a protracted schedule of four fractions delivered in a total of 18 days (Fig. 4*A*). Based on our mTOR inhibition studies, rapamycin (1 mg/kg) was delivered once every 3 days by i.p. injection throughout the course of radiation. To ensure that mTOR would be fully inhibited at the

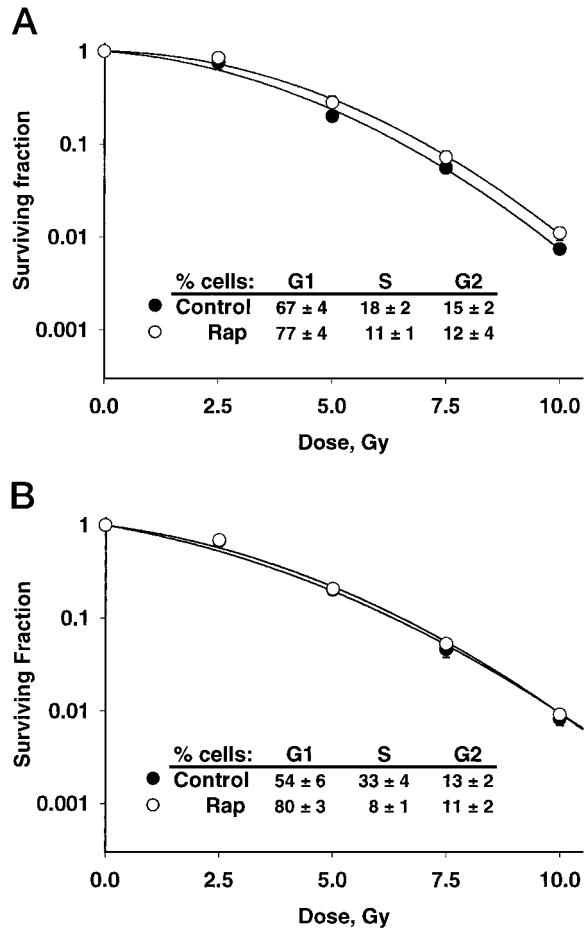

Fig. 2. Rapamycin does not affect radiosensitivity in monolayer culture. (*A*) U87 and (*B*) SKMG-3 cells were incubated for 24 h in 100 nM rapamycin or drug vehicle and then processed for flow cytometric analysis and radiation clonogenic assays. Results represent the combined data from three independent experiments, with individual data points representing the mean ± SE survival (error bars are obscured by symbols for most points). The cell cycle distribution of control and rapamycin-treated cells from these same experiments is shown (mean ± SE).

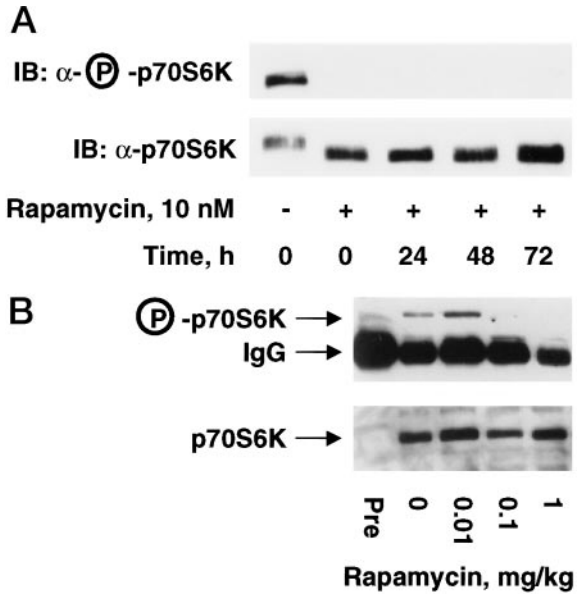

Fig. 3. Dosing schedule determination for rapamycin. *A*, U87 cells were incubated with 0 or 10 nM rapamycin for 1 h, and then the drug-containing media were washed off and replaced with fresh media. At the indicated time points after removal of the rapamycin, cells were snap-frozen and stored at −80°C. Frozen cell pellets were lysed, and p70 S6 kinase was immunoprecipitated. After SDS-PAGE and electrotransfer, nylon membranes were probed with phospho-Thr-389-specific p70 S6 kinase antisera. The membrane was stripped and reprobed with p70 S6 kinase antisera. Identical results were obtained in two independent experiments. *B*, the indicated doses of rapamycin were injected i.p. into nude mice with established U87 flank xenografts. Animals were euthanized 24 h later, and the tumors were processed as described above for determination of p70 S6 kinase phosphorylation status.

time of irradiation, rapamycin was administered 1 day before radiation. Animals with established U87 s.c. flank xenografts were randomized into four treatment groups: (*a*) control; (*b*) radiation only; (*c*) rapamycin only; and (*d*) rapamycin plus radiation. During and after treatment, tumors were measured in three dimensions, and the relative tumor volume was tracked for each animal.

The results from a representative xenograft experiment are presented in Fig. 4*B*. Identical results were obtained in a second experiment, and the data from the two experiments were pooled for an analysis of tumor regrowth delay after radiation. Control-treated tumors grew rapidly, with a volume doubling time of approximately 5 days. Compared with control, radiation therapy alone (16 Gy over 18 days) was ineffective and had no effect on tumor growth (regrowth delay of −0.2 ± 4.6 days; mean ± 95% CI). This result is consistent with reports that U87 flank xenografts are highly radioresistant; a single dose of 51.9 Gy is required to cure 50% of established flank U87 xenografts (1). The relatively low-intensity rapamycin dosing schedule also did not have significant effects on overall tumor growth rates. However, rapamycin significantly enhanced the efficacy of radiation; the combination of rapamycin with radiation resulted in a regrowth delay of 19.1 ± 6.3 days compared with treatment with rapamycin alone. Thus, combination therapy with rapamycin and radiation was significantly more effective than either radiation alone or rapamycin alone.

Rapamycin Reduces Tumor Cell Proliferation *in Vivo*. Tumor repopulation between radiation fractions also can lead to significant loss in treatment efficacy. To assess whether the dosing schedule of rapamycin used in the regrowth experiments was sufficient to inhibit tumor proliferation, the fraction of cells in S phase was determined in tumor-bearing animals treated with or without three doses of rapamycin delivered once every 3 days. Twenty-four h after the last drug dose, animals were injected with 1 mg of BrdUrd 30 min before euthanasia. BrdUrd is a thymidine analogue that is incorporated into DNA during S phase, and cells containing BrdUrd-labeled DNA were quantitated using a flow cytometric technique. Two independent experiments were performed, and the results are shown in Fig. 5. In both experiments, rapamycin treatment decreased BrdUrd labeling. Pooling the data from the two experiments for analysis, the median labeling index decreased by nearly 50% ($P = 0.02$, Wilcoxon's rank-sum test). Overall, the labeling indices were higher for both

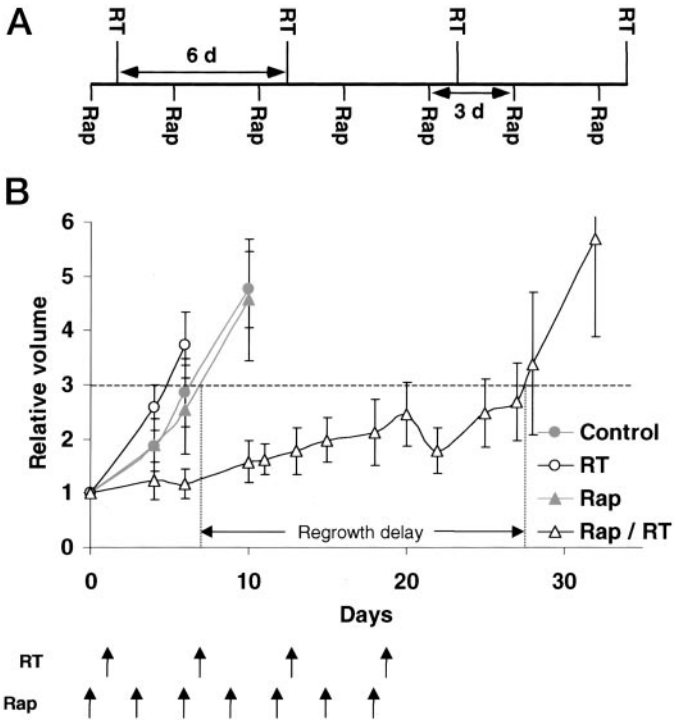

Fig. 4. Rapamycin sensitizes U87 xenografts *in vivo*. Nude mice with established U87 flank xenografts were randomized into four treatment groups: (*a*) placebo; (*b*) radiation only (4 Gy × 4); (*c*) rapamycin only (1 mg/kg); or (*d*) radiation and rapamycin. *A*, the schedule for rapamycin (*Rap*) and radiation treatment (*RT*) is depicted. *B*, the tumor regrowth for each treatment group is shown. Data points represent the mean relative tumor volume ± SE. Treatment was initiated on day 0 with the first injection of rapamycin (*Rap*). The schedule for rapamycin and radiation (*RT*) treatments is depicted below the *X* axis. Identical results were obtained in two independent experiments.

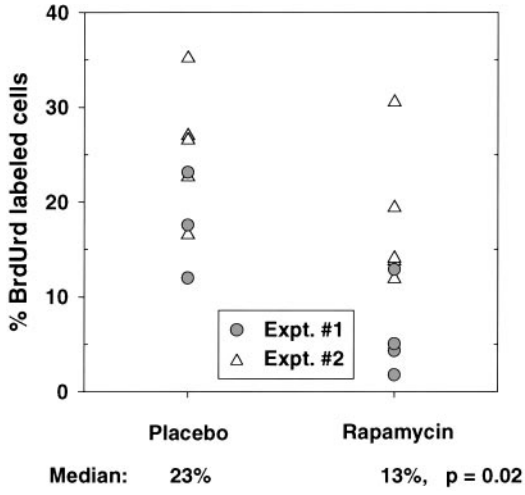

Fig. 5. Rapamycin inhibits proliferation in U87 xenografts. Nude mice with established U87 flank xenografts were treated with placebo or 1 mg/kg rapamycin i.p. on days 0, 3, and 6. On day 7, animals were injected with BrdUrd and subsequently euthanized. Tumors were then processed for flow cytometry and immunohistochemistry. The BrdUrd labeling index for individual tumors from two independent experiments is shown. Rapamycin treatment significantly decreased the labeling index in U87 xenografts.

control- and drug-treated groups in the second experiment, and this appeared to be related to tumor size. After accounting for tumor size in a multivariate statistical analysis, rapamycin treatment remained the most significant factor influencing labeling index ($P = 0.003$). Interestingly, this rapamycin-mediated cell cycle arrest in the xenografts translated into only a slightly longer tumor regrowth time for the rapamycin-treated animals (9.7 ± 2.3 days) compared with control-treated animals (8.6 ± 1.5 days). Nonetheless, these *in vivo* cell cycle arrest data are consistent with biochemical inhibition of mTOR activity by rapamycin demonstrated in Fig. 3*B* and are similar in magnitude to that seen in U87 cells after *in vitro* rapamycin treatment (Fig. 1*B*).

Rapamycin Sensitizes U87 Spheroids to Radiation. In addition to effects on tumor cell death or proliferation, rapamycin could affect host-tumor interactions, such as angiogenesis, cytokine production, or immune responses, that could influence the response to fractionated radiation. To distinguish between tumor-specific and host-specific effects of rapamycin, the response of U87 spheroids to fractionated radiation therapy was assessed in the presence and absence of rapamycin. U87 cells grown on untreated Petri dishes spontaneously form spheroids, and the response of spheroids to therapy can be tracked over time using a regrowth delay assay (26, 27). Diffusion gradients of oxygen and nutrients occur within multicellular spheroids, with low-oxygen, poor-nutrient conditions existing toward the center (28). As in solid tumors, these nutrient and oxygen gradients result in subpopulations of proliferating, quiescent, hypoxic, and anoxic tumor cells that each respond differently to radiation. Distinct from solid tumors grown in nude mice, multicellular spheroids lack key tumor components derived from host tissues that can affect treatment efficacy, such as supporting stromal cells, a vascular supply, and humoral antitumor immunity. Therefore, any effect of rapamycin on radiation response in a spheroid system must be due to the effects of rapamycin on tumor cells and would suggest that at least part of the radiosensitizing effects of rapamycin in animals are host independent.

The effects of rapamycin and radiation on spheroid growth were evaluated in regrowth delay assays that were similar to those used in the earlier animal studies. As in the xenograft studies, groups of randomly selected spheroids were treated with (*a*) vehicle control, (*b*) fractionated radiation (2 Gy × 4), (*c*) 10 mM rapamycin, or (*d*) rapamycin plus radiation. Because the volume doubling time for untreated spheroids was half that of the U87 flank xenografts, the overall length of the radiation fractionation schema was reduced to 9 days. Representative results from a single experiment are shown in Fig. 6*A*, and data from three independent experiments were pooled for statistical analysis of the regrowth delay. Similar to the animal studies, radiation alone had minimal effect on spheroid growth compared with control treatment (regrowth delay of 4.2 ± 2.6 days, mean ± 95% CI), whereas rapamycin alone had a more dramatic effect compared with control (regrowth delay = 13.5 ± 1.7 days). Presumably, the more profound growth-inhibitory effects of rapamycin in the spheroids compared with the animal studies reflect higher intratumoral drug concentrations in the spheroids *versus* the xenografts. As in the animal studies, the combination of rapamycin with radiation was significantly more effective than treatment with rapamycin alone (growth delay = 11.9 ± 3.6 days). These results are qualitatively similar to those seen in the U87 xenograft combination studies and suggest that the mechanism of rapamycin-mediated radiation "sensitization" is dependent, in part, on inhibition of mTOR signaling within tumor cells.

The animal and spheroid data are both consistent with the hypothesis that inhibition of tumor repopulation may contribute to the sensitizing effects of this drug in combination with radiation. If this is true, then another cytostatic agent with a completely distinct mechanism of action should have similar effects when combined with radiation. Therefore, we evaluated the combination of radiation with roscovitine in U87 spheroids. Roscovitine is a purine analogue that selectively inhibits CDKs 1, 2, and 5 and currently is in early clinical trials as an antitumor agent (29). In a regrowth delay assay similar to those performed previously, the effects of radiation alone on spheroid regrowth were more variable compared to control treatment with a growth delay of 15.7 ± 11.8 days (Fig. 6*B*). However, the combination of roscovitine with radiation was significantly more effective than roscovitine alone with an associated regrowth delay of 23.8 ± 7.0 days. These results are similar to those seen with the combination of rapamycin and radiation and suggest that both drugs have at least additive effects when combined with radiation in a spheroid model. Thus, inhibition of tumor proliferation during fractionated radiation therapy may enhance the efficacy of treatment, and the radiosensitizing effects of rapamycin in xenograft and spheroid models may be due partially to inhibition of tumor repopulation.

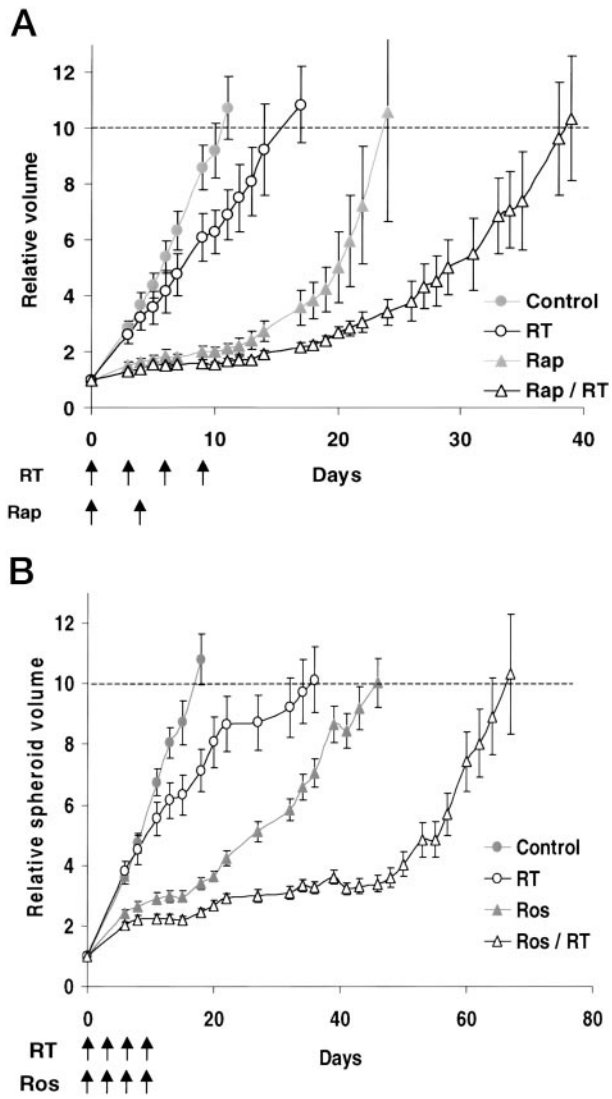

Fig. 6. Combination treatment in spheroids. Individual U87 spheroids were plated in multiwell plates and divided into four treatment groups as described in the Fig. 5 legend. In the indicated treatment groups, four fractions of 2 Gy radiation (*RT*) were delivered over a 9-day period (day 0, 3, 6, and 9). In *A*, 10 nM rapamycin was added to the culture media 30 min before radiation on day 0, and rapamycin-containing media were replenished on day 4. For roscovitine treatment (*B*), media containing 30 μM roscovitine were added 30 min before each radiation treatment (days 0, 3, 6, and 9). The regrowth for each treatment group is shown with data points representing the mean relative spheroid volume ± SE. Similar results were obtained in at least two independent experiments for each treatment condition.

DISCUSSION

Tumor proliferation during fractionated radiation is a major detrimental factor influencing local tumor control. After a dose of radiation, surviving tumor clonogens continue to proliferate and repopulate the tumor. During the latter half of a 6- or 7-week clinical course of therapy, the proliferation rate increases so that one-third to one-fourth of a typical 2-Gy radiation dose is required each day just to sterilize newly formed tumor cells (19). Moreover, tumor proliferation during extended treatment breaks is associated with a 10–15% loss in local control for each week of treatment prolongation (30). These observations suggest that pharmacological inhibition of tumor repopulation could have profound clinical impact on local tumor control. In the present study, the novel therapeutic agent rapamycin, a selective inhibitor of mTOR, was evaluated in combination with radiation both *in vitro* and *in vivo*. Rapamycin had no effect on the intrinsic radiation sensitivity of cells grown in monolayer culture, whereas rapamycin profoundly enhanced the efficacy of fractionated radiotherapy in xenografts. Although multiple mechanisms may contribute to rapamycin-mediated sensitization, the recapitulation of this phenomenon in tumor spheroids treated with either rapamycin or a selective CDK inhibitor suggests that inhibition of proliferation may contribute to this effect.

Pharmacological manipulation of tumor repopulation has been effectively combined with radiation in several model systems. We initially demonstrated the proof of principle for this concept in an estrogen-dependent MCF-7 xenograft model where tumor proliferation rates could be hormonally manipulated. In this system, removing an exogenous estrogen source during treatment slowed tumor proliferation and ameliorated the adverse effects of treatment prolongation (23, 31). The current study extends this idea by demonstrating that selective disruption of key signaling pathways important for cell growth and proliferation in U87 xenografts significantly enhances the efficacy of fractionated radiation. Similar to these results, selective inhibition of epidermal growth factor receptor signaling sensitizes squamous cell carcinoma xenografts to fractionated radiation (reviewed in Ref. 32). The observation that either rapamycin or roscovitine, a selective CDK inhibitor, enhances the efficacy of fractionated radiation therapy in an *in vitro* spheroid model bolsters the idea that inhibition of tumor proliferation can improve the efficacy of fractionated radiotherapy. Whether this effect results specifically from inhibition of tumor repopulation (tumor clonogen proliferation between radiation fractions) or from a decreased ability of tumor cells to proliferate after the completion of radiation treatment is an unresolved issue. Future experiments are planned to explore this issue by comparing the sensitizing effects of rapamycin in animals treated with radiation in a short (3-day) *versus* long (18-day) fractionation schedule.

The sensitizing effects of rapamycin in the U87 xenograft and spheroid models are quite striking in comparison with the complete lack of effect of rapamycin in the radiation clonogenic survival assays. In combination with previously published reports, these data demonstrate that rapamycin does not affect the intrinsic radiosensitivity of cells and does not act as a "classic" radiosensitizing agent (33). As discussed above, the effects of rapamycin on tumor repopulation may contribute to these disparate results. However, the *in vivo* effects of rapamycin on tumor proliferation are relatively modest (Fig. 5) compared with the profound sensitizing effects of the drug in the xenograft regrowth delay assay (Fig. 4*B*). This suggests that other factors also may be important for the sensitizing effects of rapamycin. For example, rapamycin induces significant changes in glucose and nitrogen metabolism, and the starvation-like metabolic state induced by rapamycin potentially could decrease oxygen consumption in solid tumors and improve overall tumor oxygenation (34). Any decrease in the proportion of radioresistant hypoxic cells should significantly increase the efficacy of radiation. Studies currently are under way to evaluate the effects of rapamycin on hypoxia in U87 xenografts, and the potential importance of drug-induced changes in tumor oxygenation could be evaluated by comparing the sensitizing effects of rapamycin in tumors irradiated under clamped hypoxic conditions *versus* unclamped normoxic conditions.

The present results do not exclude the possibility that rapamycin also might inhibit host-dependent processes that contribute to the profound sensitizing effects of rapamycin in the xenograft model. Rapamycin is a potent inhibitor of endothelial cell proliferation *in vitro*, and systemic administration of rapamycin can inhibit angiogenesis (35). These effects are mediated primarily through modulation of VEGF-dependent signaling, with rapamycin inhibiting both hypoxia-inducible VEGF expression and downstream signaling from the VEGF receptor (36–38). Several studies have demonstrated that angiogenesis inhibitors targeting VEGF signaling pathways sensitize tumors to radiation in xenograft systems (39–42). Taken together, these observations suggest that an antiangiogenic effect of rapamycin might enhance the efficacy of radiation. However, in the current study, rapamycin did not have a significant effect on the density of CD31-positive microvessels in established U87 xenografts (data not shown), but additional studies will be required to determine whether the rapamycin dosing schedule used was adequate to suppress VEGF expression or VEGF receptor signaling.

Molecularly targeted therapeutics are being developed against numerous cell cycle-regulatory pathways and potentially could be combined with radiation to inhibit tumor repopulation in various tumor types. To maximize radiosensitization, the selection of a therapeutic agent should be based on the identification of the operant signaling pathways driving proliferation in a specific tumor. However, cell cycle-regulatory pathways also drive stem cell repopulation in many epithelial normal tissues in response to radiation injury. Therefore, selective inhibition of repopulation should target pathways that are selectively deregulated in tumor tissues compared with normal tissues. Little is known about the molecular and cellular mechanisms responsible for radiation-induced damage in normal brain, but the paucity of rapidly proliferating tissues suggests that cell cycle-directed therapeutics may not significantly increase radiation-induced central nervous system toxicities. Selective inhibition of mTOR by the novel therapeutic rapamycin profoundly sensitizes U87 xenografts to radiation, and this demonstration provides a strong rationale for the clinical evaluation of the combination of rapamycin and radiation in patients with GBM.

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