Evaluation of the mammalian target of rapamycin pathway and the effect of rapamycin on target expression and cellular proliferation in osteosarcoma cells from dogs

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Objective—To investigate activation of the mammalian target of rapamycin (mTOR) pathway and the antitumor effect of rapamycin in canine osteosarcoma cells.

Sample Population—3 established primary canine osteosarcoma cell lines generated from naturally developing tumors.

Procedures—Expression of total and phosphorylated mTOR and p70S6 kinase was assessed by use of western blot analysis in canine osteosarcoma cells with and without the addition of rapamycin. A clonogenic assay was performed to determine the surviving fraction of osteosarcoma cells at various concentrations of rapamycin.

Results—Total and phosphorylated mTOR and p70S6 kinase expression was evident in all 3 cell lines evaluated, which was indicative of activation of this pathway. Treatment with rapamycin resulted in a time-dependent decrease in phosphorylated mTOR expression and a lack of detectable phosphorylated p70S6 kinase. No detectable change in expression of total mTOR and total p70S6 kinase was identified after rapamycin treatment. The clonogenic assay revealed a significant dose-dependent decrease in the surviving fraction for all 3 cell lines when treated with rapamycin.

Conclusions and Clinical Relevance—These data indicated that mTOR and its downstream product are present and active in canine osteosarcoma cells. The pathway can be inhibited by rapamycin, and treatment of cells with rapamycin decreased the surviving tumor cell fraction. These data support the molecular basis for further investigation into the use of mTOR inhibitors as an antineoplastic approach for dogs with osteosarcoma. (Am J Vet Res 2008;69:1079–1084)
Rapamycin is a bacterial macrolide produced by *Streptomyces hygroscopicus*. It was initially identified as an antifungal agent in a soil sample collected on the Easter Island, Rapa Nui.1,18 Subsequently, it was found to have potent immunosuppressive, antiproliferative, and antitumor effects. In 1997, rapamycin was approved by the FDA for use as an antirejection drug in kidney transplant recipients.1 It has been used clinically as an immunosuppressant for organ transplant recipients and as an antiproliferative agent in patients receiving cardiovascular stents to prevent restenosis after angioplasty.19,20 The effects of rapamycin are mediated through inhibition of the mTOR protein. Cancers in which mTOR becomes hyperactive because of PTEN mutations or overexpression of Akt are particularly susceptible to rapamycin and its analogues.1,21,22

Osteosarcoma is a common tumor in dogs, accounting for 85% of primary bone tumors and approximately 3% of all neoplasms in dogs. This tumor has an aggressive biological behavior with a metastatic rate > 90%.23-26 Current treatment recommendations are surgery (amputation or limb-sparing surgical excision) followed by chemotherapy. Median survival time with treatment is approximately 1 year, with most patients succumbing to pulmonary metastatic disease.23,26-33 Thus, finding other treatments for animals with osteosarcoma is an important endeavor. Additionally, osteosarcoma in dogs has many clinical and molecular similarities to osteosarcoma in juvenile humans, which is a rare condition. Osteosarcoma in dogs serves as a naturally developing neoplasm useful for studying this condition in humans.34,35

Alterations in the mTOR pathway have been linked to osteosarcoma. In mice, rapamycin can inhibit ezrin-mediated lung metastasis.36 Mutations in PTEN in dogs with osteosarcoma have been described,37 which suggests that dysregulation of the mTOR pathway may be a component of osteosarcomas and that mTOR may be a viable therapeutic target.

In the study reported here, we evaluated activation of the mTOR pathway in 3 canine osteosarcoma cell lines and examined the effects of rapamycin on survival in these same cell lines. Our objective was to determine whether the mTOR pathway could be effectively inhibited by rapamycin in these cells. Additionally, we assessed whether treatment with rapamycin would inhibit growth of osteosarcoma cells in tissue culture.

**Materials and Methods**

**Cell lines and primary cultures**—Three canine osteosarcoma cell lines, designated 1, 2, and 3, were derived from naturally developing tumors in clinical patients from our veterinary medical teaching hospital. The development and characterization of one of these cell lines, as well as the authentication method used for that cell line, have been described elsewhere.38 and the other 2 cell lines were derived in accordance with the same protocol. Cells were maintained in Dulbecco modified Eagle medium high glucose2 with 1-glutamine and sodium pyruvate and supplemented with 10% heat-inactivated fetal bovine serum2 and 100 U of penicillin-streptomycin/mL. Cells were grown in T-75 flasks at 37°C in a humidified environment with 5% carbon dioxide and 95% air.

**Cell preparation for assays**—Prior to all western blot experiments, cells were allowed to grow to 70% to 80% confluence. They were then washed twice with PBS solution and placed in serum-free media for 4 hours. Serum-starved cells were incubated with 0, 10, or 100nM rapamycin for 1, 4, 8, and 24 hours. Rapamycin from lyophilized powder6 was solubilized in DMSO to create a stock solution (100 µL) as per the manufacturer’s recommendations. An equivalent concentration of DMSO was added to the media of untreated and control cells.

After incubation, protein was extracted. Cells were lysed in lysis buffer containing 10mM Tris-HCl, 137mM NaCl, 10% glycerol, 1mM sodium orthovanadate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 1 µg of aprotinin/mL, 1 µg of leupeptin/mL, and 1 µg of pepstatin A/mL added. Total protein was assayed by spectrophotometry.

**Detection of proteins**—Protein aliquots (50 µg) from the cell lysates were electrophoresed in tris-acetate gels6 for evaluation of mTOR, p-mTOR, and p70S6K or in bis-tris gels6 for evaluation of p-p70S6K, which was in accordance with the manufacturer’s protocol and has been described elsewhere.39 Separated proteins were electrochemically transferred onto polyvinylidene difluoride membranes.

Membranes were blocked by incubation with 5% bovine serum albumin for 1 hour at 22°C in TBS solution (0.5 mol NaCl/L, 20 mmol Tris/L [pH, 7.5]). Then, antibodies against mTOR (diluted 1:1,000), p-mTOR (diluted 1:1,000), p70S6K (diluted 1:1,000), p70S6K (diluted 1:2,000) were added, and cells were incubated overnight with gentle agitation at 4°C in TBS solution, Tween-20, and 5% bovine serum albumin. Each membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (concentration, 1:5,000). The membrane was again washed and incubated for 5 minutes with a chemiluminescent substrate for detecting horseradish peroxidase.4 Radiographic film6 was then exposed to the membranes and developed. Each gel was subsequently stripped, and polyclonal antibodies against β actin6 were used as a positive control sample to confirm uniform total protein loading for each well.

National Institutes of Health 3T3 cells, which are known to express mTOR, were used as a positive control sample for all western blot experiments.40-42 For negative control samples, wells were loaded with all reagents, except for the protein aliquots.

**Clonogenic assay with rapamycin exposure**—Cells from tissue culture in logarithmic growth phase (70% to 80% confluent) were subjected to trypsin and counted by use of the trypan blue dye exclusion test on a hemacytometer. Cells were then transferred to 60-mm Petri dishes (initial density, 500 cells/plate) and returned to the aforementioned growth conditions, including DMSO in an amount equal to the 10nM concentration of rapamycin to control for the effects of DMSO on cell growth. Plating efficiency was determined by removing medium after 7 days of culture and staining cell colonies with crystal violet so that colonies could be manually counted. Only colonies of > 50 cells...
were counted. Cells were treated with rapamycin added to the growth medium at various concentrations (0.03 to 30nM), and cells were then allowed to grow for 7 days before staining. Colonies of > 50 cells were counted manually, and the surviving fraction was calculated. Experiments were performed in triplicate.

**Statistical analysis**—Results of the clonogenic assay were expressed as the surviving fraction, which was equal to the plating efficiency multiplied by the number of colonies counted, with the product divided by the number of cells plated. Surviving fraction for each cell line was analyzed separately. Comparisons of the surviving fractions at various rapamycin concentrations were made by the use of a Kruskal-Wallis test. Values of \( P < 0.05 \) were considered significant. Statistical analyses were performed by use of a commercially available software program.

**Results**

**Protein expression in canine osteosarcoma cells**—Analysis of western blots revealed that all 3 canine osteosarcoma cell lines expressed mTOR and p70S6K protein. The amount of total mTOR and total p70S6K proteins remained relatively constant between treated and untreated cells for each of the 3 cell lines. In addition, p-mTOR and p-p70S6K were also confirmed in all 3 cell lines, which indicated that the active form of the protein was present. Expression of these proteins was confirmed by use of molecular-weight indicators and positive control samples (Figure 1). Analysis of western blots for \( \beta \) actin confirmed uniform protein loading.

**mTOR inhibition with rapamycin**—Western blot analyses revealed that after treatment with 10 and 100nM rapamycin for 1 to 24 hours, there was continued expression of mTOR and p70S6K. Interestingly, staining intensity for p-mTOR and p-p70S6K was greater in the untreated canine osteosarcoma cells than in the 3T3 control cell line. In treated cells, the relative intensity of staining for p-mTOR decreased for each cell line, compared with staining intensity of the untreated cells from the same cell line after exposure to 10nM rapamycin. The extent of decrease in expression of p-mTOR was dependent on the duration of exposure to rapamycin and progressively decreased from 1 to 24 hours of exposure. Concentrations of 10nM rapamycin yielded results similar to those for 100nM rapamycin. There was no detectable p-p70S6K in any cell line after incubation for 1 hour or more with 10 or 100nM rapamycin (Figure 1). Analysis of western blots for \( \beta \) actin confirmed uniform total protein loading (data not shown).

**Clonogenic inhibition with rapamycin**—Results of the clonogenic assay were summarized (Figure 2). For each of the 3 osteosarcoma cell lines, there was a significant (\( P < 0.001 \)) rapamycin-induced, dose-dependent decrease in the surviving fraction. At 0.1nM rapamycin, the surviving fraction for the 3 cell lines ranged from 0.28 to 0.5. For 1nM rapamycin, the surviving fraction ranged from 0.01 to 0.21. For 30nM rapamycin, the surviving fraction ranged from 0.001 to 0.13.

During analysis of the culture plates from the clonogenic assay, it was subjectively recorded that
colonies formed on plates treated with higher doses of rapamycin were fewer in number; were more lightly stained; and consisted of smaller, less dense clusters of cells when viewed microscopically, compared with characteristics for the colonies on untreated plates and plates treated with low doses of rapamycin (Figure 3). The effect was evident for plates treated with \( \geq 1nM \) rapamycin.

**Discussion**

The study reported here was designed to evaluate the activity of mTOR in canine osteosarcoma cells and its susceptibility to inhibition with rapamycin. Analysis of results of this study indicated that mTOR is present and active in canine osteosarcoma cells. Additionally, phosphorylation of mTOR and its downstream target p70S6K can be inhibited with rapamycin. Finally, rapamycin caused a dose-dependent decrease in the surviving fraction of canine osteosarcoma cells in a clonogenic assay as well as a qualitative difference in colony growth. The variation in colony appearance at higher doses of rapamycin is of unknown relevance but indicated that in addition to decreasing the surviving fraction, rapamycin had other effects on the growth of these cells.

Limitations of the study included the small number of cell lines evaluated and the lack of a more quantitative measure of protein expression. Additionally, the correlation of clinical findings with these in vitro findings is currently unknown. This study dramatically simplified the complex interaction of multiple signaling pathways farther upstream and downstream of mTOR that may play a role in modulating the effectiveness and resistance of an in vivo tumor to mTOR inhibition.

Mammalian target of rapamycin is a central signaling molecule located downstream of Akt that integrates signals from growth factors and nutrients to regulate the cell cycle and cell growth. Signaling of mTOR is increased in many tumors in humans through upregulation of Akt or other regulatory pathways. Mutations to genes in this pathway, including PTEN, have been reported in osteosarcomas in dogs.\(^{37}\) It is not known whether there are specific mutations to those genes in the tumor cell lines used in the study reported here, but it is possible that such mutations (or the lack of such mutations) could explain some of the variation in response among cell lines. There is increasing evidence to support mTOR as a critical regulator of protein synthesis and translation initiation by phosphorylation of p70S6K and eukaryotic translation initiation factor 4E binding protein 1 (ie, 4E-BP1). Blockade of the mTOR pathway can inhibit growth of a number of tumors.\(^{39}\)

Although rapamycin has been used experimentally and clinically as an immunosuppressive agent in dogs and cats, there are minimal reported data concerning the use of mTOR inhibitors as potential antineoplastic drugs for veterinary patients.\(^{43–46}\) There has been limited evaluation of the pharmacokinetics associated with rapamycin administration and the toxic effects associated with rapamycin treatment in dogs.\(^{53,44,47}\) One concern regarding clinical use of rapamycin is the incidence of severe adverse effects when immunosuppressive doses are given to dogs. These effects include vasculitis, peritonitis, diarrhea, intussusception, oral ulcers, and emaciation.\(^{46,68}\) The frequency and severity of these adverse effects vary with the dose and duration of treatment, and the risk must be weighed against the potential benefits.

Figure 3—Photomicrographs of canine osteosarcoma cells not treated (control cells; A) or treated with 0.03nM rapamycin (B) or 10nM rapamycin (C). Because rapamycin was dissolved in DMSO, control cells were treated with an equal concentration of DMSO (ie, 0.03 and 10nM). Cells were allowed to grow for 7 days, and then they were fixed with ethanol and stained with crystal violet. Notice that cells from plates with a higher rapamycin concentration formed smaller colonies composed of less densely arranged cells. Bar = 500 \( \mu \)m.
effects are inversely proportional to the dosage in the range of 0.3 to 1.5 mg/kg/d. No substantial adverse effects were reported when alternate-day administration of 0.1 mg/kg was used.44

A maximally tolerated dose for rapamycin in dogs has not been determined; however, published data indicate that injection at a rate of 0.05 mg/kg/d appears to be tolerated.45 This results in whole blood concentrations of 9 to 14 μg/L, which corresponds to 9.6 to 15.3 nM.43 There appears to be variation in oral bioavailability in dogs and humans, but high serum concentrations can be achieved.49 A study designed to use dose escalation to determine bioavailability, pharmacokinetics, and the tolerated dosage in dogs would be needed to verify that rapamycin can be administered safely and achieve blood concentrations that are potentially therapeutic.

For the use of rapamycin in humans, it has been determined that the dose needed to inhibit mTOR in patients with tumors is much lower than the dose needed for immunosuppression in transplant recipients. Additionally, current data from ongoing clinical trials indicate that mTOR-targeted treatment with rapamycin and its derivatives is tolerated well and that there is substantial clinical activity in human patients with several types of malignancies, including melanoma, mantle cell lymphoma, non-Hodgkin's lymphoma, multiple myeloma, non–small-cell lung tumors, and renal cell carcinoma.50,51 Synergism between mTOR inhibitors and radiotherapy and chemotherapeutics (including paclitaxel and carboplatin) is also extremely promising.52–56 To our knowledge, there are no published reports describing the mTOR protein and the effects of rapamycin in a canine tumor cell line. With inhibition of mTOR emerging as a promising novel therapeutic target in the treatment of humans with various malignancies, there is potentially substantial direct clinical and comparative benefit to evaluating this pathway in dogs with naturally developing tumors.

References


