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The Effect of Rapamycin on Bone Growth in Rabbits

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Abstract

mTOR is a nutrient-sensing protein kinase that regulates numerous cellular processes. Our prior studies using the mTOR inhibitor, rapamycin, indicate an important role for mTOR in chondrogenesis. We extended our observations to a physiological, in vivo model of bone growth, direct infusion of rapamycin into the proximal tibial growth plates of rabbits. Rapamycin or DMSO vehicle was infused directly into growth plates by an osmotic minipump for 8 weeks. Tibial growth was followed radiographically. At the end of the experiment, growth plates were recovered for histological analysis. Six animals were studied. No untoward effects of rapamycin infusion were found. Bone growth of limbs exposed to rapamycin was slower than control limbs, particularly during the period of most rapid growth. Histological analysis revealed that growth plate height in the rapamycin-infused limbs was reduced. Both the hypertrophic and proliferative zones were significantly smaller in the rapamycin-infused limbs. Direct infusion of rapamycin into proximal tibial growth plates decreased the size of the growth plate and inhibited overall long bone growth. Rapamycin appears to affect both the proliferative and hypertrophic zones of the tibial growth plate. Our results indicate that nutrients may exert a direct effect on long bone growth via mTOR-mediated modulation of chondrogenesis at the growth plate, and suggest that the possible inhibitory effects of rapamycin on skeletal growth warrant further attention before its use in children.

Keywords

growth plate; chondrocytes; mTOR; cell proliferation; cell differentiation

Chondrocyte growth and differentiation are essential to long bone growth.^{1,2} The chondrocytes that reside in the epiphyseal growth plate undergo a series of choreographed changes that take them from an undifferentiated, proliferative state to terminal differentiation. During this process, cell number increases as chondrocytes proliferate, cell size increases, and the chondrocytes acquire the ability to synthesize matrix proteins. Eventually, chondrocytes undergo apoptosis. Chondrocyte proliferation and hypertrophy correlate with the rate of bone elongation. Disorders that disrupt proliferation and differentiation manifest as decreased linear growth.

The mammalian Target of Rapamycin (mTOR) is a nutrient-sensing protein kinase that regulates numerous cellular processes, including ribosome biogenesis, translation, transcription, and autophagy.^{3–6} Rapamycin is a highly specific inhibitor of mTOR.⁶ Its target is a particular mTOR-containing complex, TORC1.⁷ This drug is widely used as an

immunosuppressant agent in organ-transplanted patients. In addition, its cell growth suppression properties have led to its use as a chemotherapeutic agent for a variety of adult cancers.^{8,9} More recently, the use of rapamycin has been expanded to children. Its effect on long bones has not been reported, likely due to limited usage in children.

In addition to its role as a therapeutic agent, rapamycin can be used to assess the role of the mTOR nutrient signaling pathway on cell growth, proliferation, and differentiation. The basis for this is the well defined role of the TORC1 complex, the rapamycin-sensitive mTOR complex, in detecting amino acid sufficiency and overall energy sufficiency of the cell through the upstream signaling of AMP kinase.^{10,11}

We previously exploited the specificity of rapamycin for mTOR to examine the role of this signaling kinase in chondrogenesis.¹² Our findings in the ATDC5 chondrogenic cell line indicated that mTOR signaling contributes to chondrocyte hypertrophy and differentiation. We showed similar effects using fetal rat metatarsal explants. The present study was undertaken to extend our studies to a physiological system to examine the effects of mTOR inhibition on the growth plate and on long bone growth using a model similar to human long bone growth: tibial growth in the rabbit. We accomplished this through long-term infusion of rapamycin into proximal growth plates using implantable osmotic pumps. Our results support the conclusion that signaling via mTOR is involved in growth plate chondrogenesis. By extension, prolonged clinical use of rapamycin may directly affect long bone growth via its effect on growth plate chondrocytes.

MATERIALS AND METHODS

Animals and Surgical Procedures

Five-week-old New Zealand White rabbits were purchased from Millbrook Breeding Labs (Amherst, MA) and housed for 1 week prior to surgery. The animals received NIH Open Formula Rabbit Rations (NIH 32) and water at libitum. At 6 weeks of age, they were premedicated with intramuscular buprenorphine (0.03 mg/kg), xylazine (6 mg/kg), and ketamine hydrochloride (20 mg/kg). One intravenous dose of Cefazolin, 20 mg/kg, was given for infection prophylaxis. The rabbits were then intubated and placed under general anesthesia with isoflurane. Cardiorespiratory status was monitored throughout the procedure.

Both rabbit hind limbs were shaved and prepared with betadine. A 10-gauge intravenous needle was passed percutaneously from medial to lateral through the proximal tibial growth plate under fluoroscopic guidance. The needle was placed in line with the growth plate in the coronal plane and at the midpoint of the AP width of the growth plate in the sagittal plane. One-centimeter longitudinal skin incisions that were centered on the needle entry and exit points were made. An attachable vinyl catheter was passed through the needle from lateral to medial, with the open end of the catheter entering the point of the needle. The needle was then withdrawn from the medial side, leaving the vinyl catheter in the growth plate. The vinyl catheter was advanced medially, allowing the fenestrated segment to rest on the growth plate. The tail end of the catheter, resting against the lateral aspect of the tibia, was sutured to the iliotibial band with 3-0 Vicryl suture. A 1-cm incision was made in the right inguinal canal region, and a subcutaneous pocket was developed. The open end of the catheter was tunneled subcutaneously to this pocket and attached to the prepared osmotic pump. The pump was placed in the pocket. All skin incisions were closed with buried, interrupted subcutaneous 3-0 Vicryl sutures.

Alzet osmotic pumps (200- μ L capacity with a pump rate of 0.25 μ L/h for 4 weeks) were used. Pumps were loaded with sterile 200 ng/ μ mL rapamycin (LC Laboratories, Woburn, MA) diluted in 5% DMSO (Sigma, St. Louis, MO) for right limbs and 5% DMSO alone for left

limbs. The pumps were primed in a saline bath for 24 h prior to implantation. Pumps were replaced at the end of 4 weeks, and the experiments were terminated at the end of 8 weeks.

Two metal pins (Kirschner wire, Synthes USA, West Chester, PA) were inserted parallel to the proximal tibial growth plate. One pin was placed in the bony metaphysis about 5 mm distal to the growth plate, and the second was inserted 5 mm distal to the first. Postoperative analgesia with buprenorphine (0.03 mg/kg) was given every 12 h during the first 3 postoperative days.

Serial radiographs of the tibia were obtained at weekly intervals. The rabbits were sedated with an intramuscular injection of xylazine (6 mg/kg) and ketamine hydrochloride (20 mg/kg). Rabbits were placed prone on an X-ray cassette labeled with radiopaque distance reference markers. The hind legs were fixed in neutral rotation and full extension with tape. The X-ray source was positioned 90 cm above the cassette. The radiographs were examined on a light board by an observer blinded to the treatment. The distance from the proximal tibial plateau to the proximal edge of the wire marker was measured using a micrometer along the central axis of the tibia. We also measured the long axis distance between the tibial plateau to the first pin, the distance between the first and second pin, and the distance between the distal pin and distal tibial epiphysis. The observer making the measurements was blinded as to which side received rapamycin or DMSO vehicle.

One rabbit was randomly chosen 4 weeks after implantation and euthanized with sodium pentobarbital (100 mg/kg). The remaining rabbits were euthanized at 8 weeks using the same method. The proximal tibia was harvested and placed in 4% paraformaldehyde for 48 h.

All experimental procedures on the rabbits were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital.

Qualitative and Quantitative Histology and Histologic Staining

The proximal tibial specimens were decalcified in Richman-Gelfand-Hill solution for 16 h, washed with tap water for 8 h, dehydrated with serial ethanol and xylene washes, then embedded in paraffin. Samples were sectioned at three evenly distributed points along the medial-lateral extent of the tibia. Multiple 6- μ m slices were obtained from each cut surface and deparaffinized. The sections were stained with hematoxylin and eosin as well as safranin O.

Growth plate height was measured at the 8-week time point using a digital photomicrograph. The resting zone (R) and proliferative zone (P) heights were combined for a single measurement, because the transition between these two zone was often indistinct. The hypertrophic zone (H) was measured from the first chondrocyte with a height of $>10\text{ }\mu\text{m}$ to the last intact lacuna with a hypertrophic cell, as previously described.¹³ Total growth plate height was defined as the distance from the start of the resting zone to the last intact lacuna along a column and was estimated as the sum of the R + P and H measures. Measurements were made along the longitudinal axis of the growth plate columns. Two slides from the central sagittal section of each leg were examined, and five evenly distributed areas from each slide were measured. The measurements were then averaged to obtain a single value for each growth plate.

Statistical Methods

The accumulated distance between the tibial plateau and the first pin was the primary indicator of bone growth. The distance between the first pin and second was expected to be constant with time because it did not span a growth plate. Measurements were made in duplicate from each radiograph, and the results were averaged for a single measurement per bone. All duplicate measurements were within 0.2 mm of one another. Data are expressed as the mean and standard

deviation. Data were analyzed by repeated-measure analysis of variance (ANOVA) and *t*-test as was appropriate to the particular analysis.

RESULTS

All the animals tolerated the growth plate infusion of rapamycin or DMSO vehicle without any apparent side effects. The mean weight of the rabbits was 1.28 kg at the beginning of the experiment and 2.66 kg when the study was terminated. Average weight gain was comparable to that seen in unmanipulated rabbits (data not shown).

Infusion of rapamycin was associated with a smaller increase in bone length from baseline relative to the contralateral bones that were infused with DMSO alone (Fig. 1). The incremental length for rapamycin infused limbs and control limbs began to diverge after 3 weeks ($p = 0.08$). The difference was maximal at 4 weeks ($p = 0.015$) and remained significant until 6 weeks ($p = 0.05$). No statistically significant difference existed at 8 weeks ($p = 0.38$) when the study was terminated.

The most rapid rate of growth occurred during the first 4 weeks. During this time, the rate of growth in the control group was higher than the rapamycin group ($p < 0.05$). After 4 weeks, the growth rate began to decelerate in both groups. Thus, the power of the analysis was greatest at a time when growth rate was maximal. These results were interpreted as indicating that the infusion of rapamycin into the proximal tibial growth plate inhibited long bone growth.

At the end of the 8-week infusion period, a histomorphometric analysis showed that the mean total growth plate heights for the rapamycin-infused and control groups were significantly different (Fig. 2A). A paired analysis in which each animal was used as its own control, an approach based on the experimental design in which contralateral limbs were studied, showed that rapamycin induced a consistent significant ($p < 0.05$) decrease in growth plate height in all animals (Fig. 2B). One animal was excluded from these data because closure of the growth plate had occurred on the rapamycin infused side but not the control side.

The four animals included in the paired analysis were analyzed further to assess the specificity of the rapamycin effect for zones within the growth plate (Fig. 3). The mean size of the proliferative zone at 8 weeks was $170 \pm 17 \mu\text{m}$ for the DMSO-infused growth plates and $154 \pm 21 \mu\text{m}$ for the rapamycin-infused growth plates ($p < 0.05$). The mean size of the hypertrophic zone at 8 weeks was $158 \pm 16 \mu\text{m}$ for the DMSO-infused growth plates and $134 \pm 23 \mu\text{m}$ for the rapamycin-infused growth plates ($p < 0.05$).

Qualitative histological analysis was carried out for all growth plates using H&E and Safranin-O staining (Fig. 4). The control growth plates demonstrated the normal, columnar arrangement of cells, with hypertrophic cells packed neatly. In contrast, the rapamycin-infused growth plates appeared to have fewer cells per column. This was confirmed by cell counting, which showed 12 ± 4 cells per column in the DMSO-infused growth plates versus 7 ± 3 cells in the rapamycin-infused growth plates ($p < 0.05$). Although not immediately apparent in individual photomicrographs, quantifying the intensity of the red Safranin-O staining, which correlates with proteoglycan content, showed a modest reduction in the rapamycin-infused growth plates relative to the DMSO-infused controls. Images from rabbit 5, which showed complete fusion of the rapamycin-infused growth plate, are shown at the bottom of the figure.

DISCUSSION

mTOR is well established as a key regulator of ribosome biogenesis, global protein synthesis, translation control, and gene expression.^{14,15} The direct phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein 1 provides for the regulation of cap-dependent

translation. Ribosomal protein S6 kinase is another target of mTOR, although its functional significance is less certain. Although considerable attention has been paid to the mechanisms by which mTOR exerts other effects on cell cycle progression, gene expression, and apoptosis, precise mechanisms have eluded identification.

The activity of mTOR is regulated by many factors including nutrients, growth factors, and energy metabolism.^{16–18} We hypothesized that mTOR may function as a regulator of chondrocyte proliferation and/or differentiation, thereby allowing for the direct regulation of growth by nutrient abundance. The effect of nutritional status on long bone growth is well documented.^{19,20} Inadequate nutrition is associated with diminished long bone growth, whereas overnutrition accelerates growth in children with obesity.²¹ However, most investigators who have studied the effect of nutritional status on growth have focused on the IGF-I/GH axis.²² Our studies^{23,24} demonstrated that insulin, in addition to IGF-I, has a direct role in chondrocyte differentiation and long bone growth, thus providing an additional mechanism by which nutrition can modulate bone growth. Given that mTOR is regulated by growth factors, including insulin, and by nutrients, most notably, amino acids,²⁵ we consider it likely that modulation of mTOR activity affects long bone growth via both indirect (insulin and IGF-I) and direct (nutrient) effects.

As noted above, we recently characterized the role of mTOR in chondrocyte growth and differentiation using the ATDC5 chondrogenic cell line and fetal rat metatarsal explants.¹² Our findings indicate that mTOR signals to chondrocyte proliferation and differentiation, although rapamycin-induced mTOR inhibition has a more pronounced effect on the latter. In the present study, we expanded our findings to a physiological, in vivo model of long bone growth. By infusing rapamycin directly into the rabbit tibial growth plate, we could assess the effect of mTOR inhibition in a model similar to humans.

At the dosage of rapamycin used in this study, the rabbits appeared well. They ate well and gained weight normally, thus making the confounding effects of altered nutrition or hormonal status unlikely contributors to our observations. By using the contralateral limb for the control (vehicle) infusion, we reduced interanimal variability. Nonetheless, we did not observe any systemic effects of rapamycin. The tibial growth rate was most rapid in the first 4 weeks of the experiment, consistent with prior observations.²⁶ The inhibitory effect of rapamycin on linear bone growth was most pronounced at the peak growth rate during the fourth week of infusion. At the end of the infusion period, the effect on growth was not significant. The period beyond the fourth week coincided with a declining growth rate, possibly suggesting that growth acceleration and deceleration may be regulated by different mechanisms. Another possible explanation is that the effect of rapamycin wanes due to adaptation of cells to its inhibitory effect on mTOR. Finally, another mechanism might intervene, resulting in the kind of unilateral catch-up growth that was observed by Baron and coworkers²⁶ on the effect of glucocorticoid on bone growth.

Examination of the growth plates in our animals showed that the proliferative and hypertrophic zones were proportionally smaller in rapamycin-exposed limbs. The smaller proliferative zone would likely contribute to attenuated bone growth. Under normal conditions in the rat, each chondroblast undergoes approximately four to five rounds of cell division. Each chondroblast, through repeated division, gives rise to about 30 hypertrophic chondrocytes, which contributes significantly to linear growth.²⁷ BrdU incorporation of the chondrocytes as reported by Schrier et al.²⁸ has been shown to be a useful tool. We attempted performing this measurement, but were unsuccessful. A possible explanation for our findings is that rapamycin also affects the number of cells that enter the proliferative zone. This explanation is also consistent with the observation that the lengths of both the proliferative and hypertrophic zones were reduced in rapamycin-infused growth plates, and that the number of cells in each column of the growth

plate was reduced in association with rapamycin infusion. Rapamycin-infused growth plates also showed a smaller hypertrophic zone, indicating an effect on chondrocyte hypertrophy. This may relate to the ability of mTOR to regulate cell size.²⁹ It may also reflect an effect of mTOR on chondrocyte differentiation, something we have observed in both cell culture and explant systems.¹² In our prior cell culture and explant studies, we observed that rapamycin affects the expression of Indian Hedgehog (Ihh), a key regulator of chondrocyte differentiation.² Unfortunately, we were unable to perform immunohistochemistry for Ihh on the rabbit growth plate because available antibodies are rabbit-derived. When we studied the effect of rapamycin on chondrocyte apoptosis, rapamycin did not seem to increase chondrocyte cell death, similar to our observation in fetal rat metatarsal bones (manuscript in preparation).

Rapamycin is being used widely as an immunosuppressant and chemotherapeutic agent with more than 70 clinical trials in progress. The use in the pediatric population is on the rise. Although effects on skeletal growth have not been noted so far, this may be a function of the small numbers of study subjects and the use of the drug under circumstances that can impair linear growth.³⁰ The result of our study suggest that the possible effects of rapamycin on long bone growth in children warrant attention.

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Figure 1.

Cumulative proximal tibial growth of limbs infused with DMSO (●) or rapamycin (○). Data are shown as the mean $\pm$ SEM for $n = 6$ per group until week 4 and $n = 5$ from weeks 4 to 8. * $p = 0.08$, ** $p = 0.015$, *** $p = 0.05$.

Figure 2.

Effect of rapamycin infusion on the height of the proximal tibial growth plate. The growth plate height was determined at the end of week 8. (A) Data for four animals per group are shown as the mean + SD. * $p < 0.05$ versus DMSO. (B) The effect of rapamycin is shown for each individual animal ($n = 4$). One animal was excluded because the growth plate was fused in the rapamycin treated limb. Each symbol represents an animal. The graph compared mean height of the growth plate + SE of each animal. DMSO was infused to the left limbs and rapamycin was infused to the right limbs. $p < 0.05$.

Figure 3. Histological analysis of DMSO-infused and rapamycin-infused growth plates. The heights of the proliferative zone (A) and hypertrophic zone (B) are shown as the mean + SD for four animals. * $p < 0.05$.

Figure 4.

Representative photomicrographs of sagittal sections of DMSO-infused (left) and rapamycin-infused (right) proximal tibial growth plates stained for H&E (top 2 panels at 1× and 10× magnification) and Safranin-O (bottom 2 panels at 1× and 10× magnification). The lower panels show representative photomicrographs from rabbit 5, which showed fusion of the rapamycin-infused growth plate.