Low Concentration of Rapamycin Inhibits Hemangioma Endothelial Cell Proliferation, Migration, and Vascular Tumor Formation in Mice

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ABSTRACT

Background: Vascular endothelial cell excessive proliferation is the main biological behavior of hemangioma. Rapamycin regulates the growth of endothelial cells by inhibiting mammalian target of rapamycin (mTOR). Thus hemangioma accompanied by excessive mTOR activation should be sensitive to rapamycin. We aimed to illustrate the effect of low-concentration rapamycin on hemangioma and provide a safe and effective drug therapy.
Methods: Mouse hemangioendothelioma endothelial cells and Nu/Nu mice were used. Rapamycin was applied in a concentration from 1 nM to 20 nM. WST-1 cell proliferation and transwell migration assays were used to analyze vascular tumor proliferation and migration in vitro. Xenograft mouse models were used to test vascular tumor growth in vivo.
Results: Low-concentration rapamycin (1 nM) inhibited hemangioendothelioma endothelial cell proliferation and migration in vitro and vascular tumor growth in vivo. The mechanism was decreased activation of the protein kinase B/mTOR/S6 ribosomal protein (S6) signaling pathway.
Conclusions: Rapamycin used in vitro was analogous to low serum concentration rapamycin (7–16 nM) and also significantly inhibited the growth of hemangioma. These results demonstrate a low-toxic drug therapy for hemangioma and encourage continued development of rapamycin and its analogs for use in vascular tumor therapy.

Introduction

A hemangioma is a congenital anomaly composed of a mass of blood vessels that resemble a tumor with a variety of clinical manifestations.[1–3] So far, the cellular and biomolecular mechanisms of hemangioma formation are unclear. The treatment of hemangioma mainly involves hormone therapy, chemical therapy, laser ablation, and surgical resection.[4] These methods lead to side effects of varying degrees, especially in children. Rapamycin is an antifungal, antineoplastic, and antibacterial macrolide drug. By binding to the cytosolic protein FK-binding protein 12, it inhibits the function of the mammalian target of rapamycin (mTOR), which is a specific cell regulatory protein responsible for eukaryotic cell and tumor cell growth. Rapamycin not only shows a strong effect of immune suppression, but also prevents postangioplasty coronary artery restenosis. It can also be used in a wide range of antineoplastic therapies.[5–7] As a specific mTOR inhibitor, it has been used as a chemical activator in autophagy research both in vitro and in vivo.[8] We aimed to illustrate the effect of low-concentration rapamycin on the growth and proliferation of hemangioma, and provide a safe and effective drug therapy.

Methods

All the animal work described in this article was carried out in accordance with EC Directive 86/609/EEC for animal experiments.

Cell line and culture maintenance

Mouse hemangioendothelioma endothelial (EOMA) cells[9] were used. EOMA cells were adapted for growth in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS) and antibiotics. Rapamycin was diluted into 2.5 mg/mL in dimethylsulfoxide and stored at −20°C. The work concentrations of rapamycin were diluted in

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DMEM (from 1–20 nM). EOMA cells of serum-starved were cultivated in DMEM with 1% FBS for 18 hours. EOMA cells of serum group were cultured in a full DMEM medium. Rapamycin was applied to EOMA cells overnight in a gradually increasing concentration (from 1–20 nM).

Immunoblotting

A standardized protocol was used to analyze the phosphorylation of the protein kinase B (AKT)/mTOR signaling pathway in EOMA cells treated with rapamycin. EOMA cells cultured in a 60-mm plate were treated with 10 nM rapamycin. After 48 hours, scraped cell lysates were harvested. Western blot analysis was performed in triplicate as described elsewhere.[10] All antibodies, including phospho-AKT (Ser473), phospho-mTOR, phospho-glycogen synthase kinase-3 beta (GSK3β), and phospho-S6 Ribosomal Protein (S6) were purchased from Cell Signaling Technology (Danvers, MA, United States). α-Tubulin was purchased from Calbiochem (San Diego, CA, United States).

WST-1 cell proliferation assay

EOMA cells (1×10^3 per well) were plated in a 96-well plate in complete medium. The medium was replaced the following day with fresh medium with and without rapamycin. EOMA cells were then cultured for 48 hours. Proliferation was assayed using a WST-1 assay. WST-1 reagent (10 μL) was added to each well and EOMA cells were incubated again for another 4 hours. The absorbency of the samples was measured against a background control as blank at 450 nm using a microplate reader according to the manufacturer's protocol.

Cell migration assay

Transwell cell inserts are from Corning (Tewksbury, MA, United States) with 8-μm pores were used. Growth factor-reduced matrigel (100 μL) was first diluted to 1 mg/mL by serum free cold DMEM and then added into the upper chamber of a Transwell support and incubated for 4 hours for gelling at 37°C and 5% carbon dioxide. EOMA cells (100 μL) at < 50% confluence were pretreated with and without rapamycin in normal growth medium for up to 48 hours before seeding into the cell inserts. The lower chamber was filled with medium containing a chemoattractant (ie, vascular endothelial growth factor [VEGF] 10 ng/mL). The cells were then incubated for 24 hours. Unmigrated EOMA cells were scraped from the upper side of the inserts, and the migrated EOMA cells were stained with Diff-Quick Stain kit was from IHCWorld(Woodstock, MD, United States) and counted under an inverted microscope. The data were expressed as the average of 4 high-power fields of triplicate samples after subtraction of the average of cells migrating toward medium without chemoattractant.

Animals and tumor growth

Four- to 6-week old female Nu/Nu mice were used in our experiments. Vascular tumor model was established by subcutaneous injection. EOMA cells were injected subcutaneously in the flank of mice (2×10^5 cells per site, 2 sites per mouse, 5 mice per group). Rapamycin or vehicle was injected daily intraperitoneally. The mice were under close observation daily to monitor their general condition and measure the tumor size with a caliper. The mice were then sacrificed after 2 weeks of treatment with rapamycin.

Statistical analysis

All results are expressed as mean (SD). The statistical significance of differences was assessed by using the Statistical Package for the Social Sciences (IBM-SPSS Inc IBM SPSS Statistics 21.0-(2012.Aug), Armonk, New York). The data were analyzed using a 2-tailed Student t test. The differences between the means were considered significant at $P < 0.05$.

Results

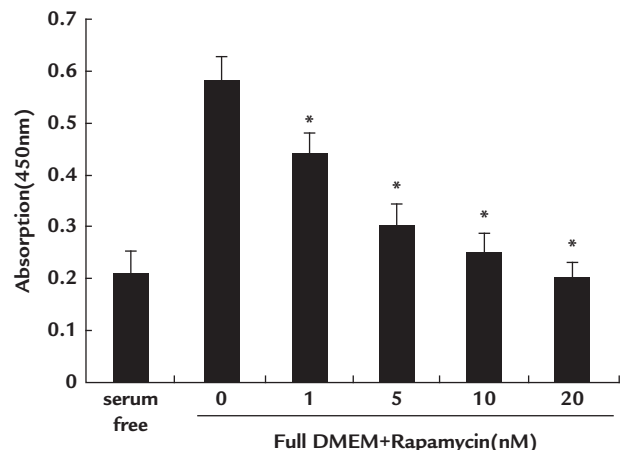### Low-concentration rapamycin inhibited EOMA cell proliferation in vitro

To analyze the effect of rapamycin on vascular tumor growth in vitro, EOMA cells were incubated in different concentrations of rapamycin. WST-1 cell proliferation assay were used to measure cell proliferation. Quadruplicate cultures of EOMA cells were grown overnight in complete medium with or without rapamycin. Rapamycin inhibited proliferation of EOMA cells in a dose-dependent manner (**Figure 1**). A concentration > 1 nM rapamycin showed a significant inhibition of the proliferation (0 nM vs 1, 5, 10, and 20 nM; $P < 0.01$). These results showed that EOMA cells have obvious sensitivity to rapamycin. Different concentrations of rapamycin all played an important role in the inhibition of EOMA cell proliferation (**Figure 2**). More significantly, the low concentration group, compared with controls, still showed significant inhibition with minimal side effects.

### Low-concentration rapamycin inhibited EOMA cell migration in vitro

The ability of tumor cells to migrate is an important prerequisite for tumor dissemination and metastasis. The ability of rapamycin to inhibit the migration of EOMA cells toward VEGF was measured using transwell migration assays. Forty-eight hour low-dose rapamycin treatment showed a significantly strong inhibitory effect on EOMA cells. These antimigratory effects of rapamycin were also dose-dependent (**Figures 3** and **4**) ($P < 0.01$).

Low-concentration rapamycin inhibited vascular tumor growth in vivo

The effect of rapamycin on vascular tumor growth in vivo was tested next. We used xenograft mouse models with EOMA cells injected subcutaneously in the flank of mice. Mice of the control group were given normal saline by intraperitoneal injection, whereas those of the rapamycin test group were given a dose of

Figure 1. Low concentration of rapamycin treatment affected hemangioendothelioma endothelial (EOMA) cell proliferation (n = 4 in each group). Rapamycin (1–20 nM) all inhibited EOMA cell proliferation compared with serum control ($P < 0.05$). DMEM = Dulbecco's modified Eagle's medium.
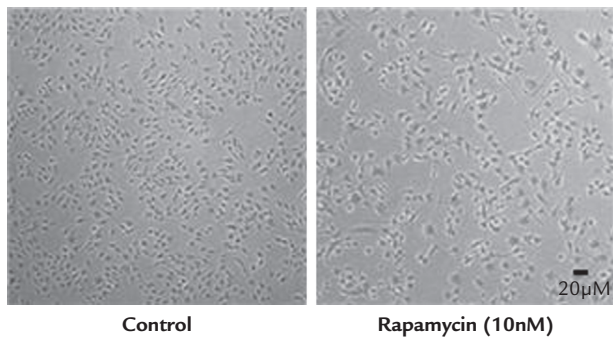
Figure 2. Single-layer hemangioendothelioma endothelial (EOMA) cells observed under microscopy (20 ×) with rapamycin treatment for 48 hours. Rapamycin (10 nM) inhibited EOMA cell proliferation compared with control.
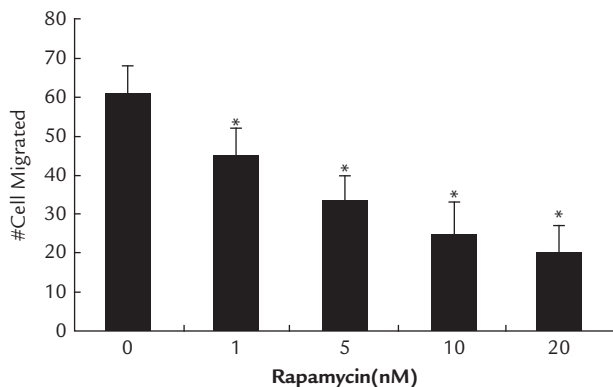
rapamycin (0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, or 2 mg/kg) daily. The volume of vascular tumor was measured from Day 0 to Day 12. Compared with the control group, vascular tumor size in the rapamycin test group (0.5 mg/kg) was much smaller from Day 6 to Day 12. This low dose of rapamycin blocked vascular tumor growth significantly (**Figure 5**) ($P < 0.05$). The mice in the highest dose cohort died.

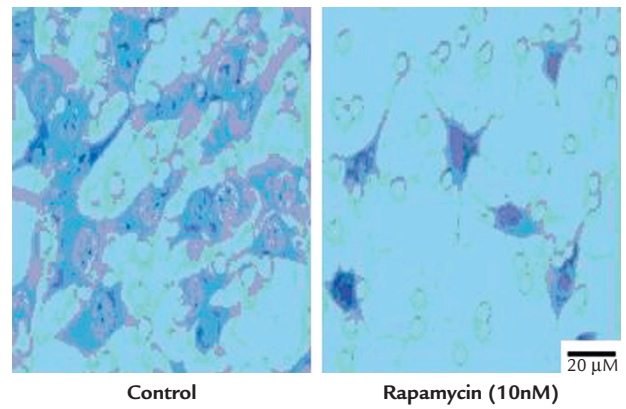*Rapamycin inhibited phosphorylation of the AKT/mTOR/S6 signaling pathway*

We next examined whether AKT/mTOR/S6 signaling activation was also inhibited and attempted to find the probable mechanism by which rapamycin inhibits hemangioma endothelial cell proliferation, migration, and vascular tumor formation. In the experiments above, 10 nM rapamycin showed obvious inhibitory effect on proliferation, migration, and vascular tumor growth in EOMA cells. Here, EOMA cells were treated with 10 nM rapamycin for 48 hours. EOMA cells of the starvation group were treated with DMEM medium with only 0.1% FBS. EOMA cells of the serum group were treated with full DMEM with 10% FBS. Western blot analysis showed that phosphorylation of AKT (Ser473), mTOR, GSK3β, and S6 were all hyperactivated in the serum group, but markedly decreased by rapamycin treatment with α-tubulin as protein loading control (**Figure 6**).

Discussion

Hemangiomas are formed by an abnormal collection of blood vessels that may resemble a tumor. They are usually found on the skin or the internal organs and can lead to disfigurement and/or

Figure 4. Migrated hemangioendothelioma endothelial (EOMA) cells with rapamycin treatment for 48 hours under inverted microscope (200 ×). Rapamycin (10 nM) inhibited EOMA cell migration compared with control.

life-threatening consequences.[11] Hemangioma is implicated in a wide spectrum of clinical diseases, ranging from common hemangioma in children to malignant angiosarcoma in adults.[12] Vascular tumor malformations have been found in some genetic syndromes, like posterior fossa malformations–hemangiomas–arterial anomalies–cardiac defects–eye abnormalities–sternal cleft and supraumbilical raphe syndrome, Kasabach-Merritt syndrome, Sturge-Weber syndrome, and Klippel-Trenaunay syndrome.[1,13,14] Treatment options include laser ablation, surgical resection, and medication. Drugs such as propranolol, interferons, and corticosteroids are commonly used.[15,16] However the efficacy and risks of these drugs are still under investigation due to varying severity of the side effects, including the possible risk of interferon causing neurologic problems in children.

Rapamycin is a bacterial macrolide previously identified as an antifungal agent. It is also an effective AKT inhibitor in vascular endothelial cells.[17,18] It inhibits target of rapamycin (TOR) proteins, a highly conserved group of serine/threonine kinases.[15] mTOR
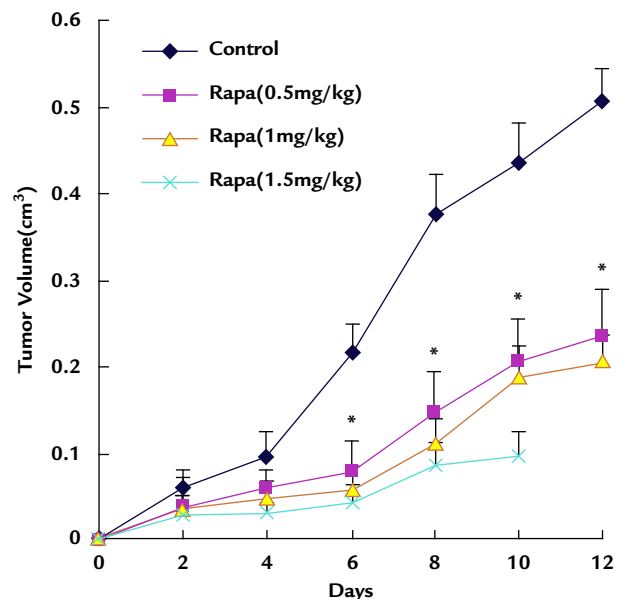

Figure 3. Low concentrations of rapamycin treatment affected cell migration (n= 5 in each group). Rapamycin (1–20 nM) inhibited hemangioendothelioma endothelial cell migration compared with control ($P < 0.05$).

Figure 5. Rapamycin treatment affected tumor growth in Nu/Nu mice. hemangioendothelioma endothelial cells were injected subcutaneously in the flank of mice (5 mice per group). Rapamycin (0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, and 2 mg/kg) or vehicle was injected daily, intraperitoneally. Tumor size was monitored for 12 days. Low concentration of rapamycin (0.5 mg/kg) significantly reduced tumor growth compared with control from Day 6 to Day 12 ($P < 0.05$). The mice in the highest-dose group (2 mg/kg) died.
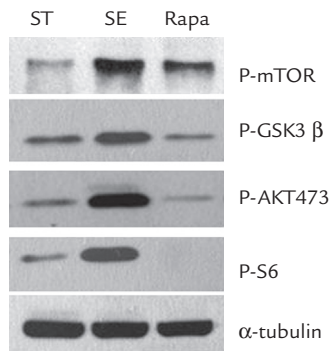
Figure 6. Western blot analysis of rapamycin treatment in hemangioendothelioma endothelial cells for protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. Decreased phosphorylations of mTOR, AKTSer473, glycogen synthase kinase-3 beta (GSK3β), and S6 occurred with α-tubulin as protein loading control. ST = starvation group treated with Dulbecco's modified Eagle's medium with only 0.1% fetal bovine serum; SE = serum group treated with full Dulbecco's modified Eagle's medium with 10% fetal bovine serum; Rapa = 10 nM rapamycin for 48 hours.

regulates phosphorylation of mTOR effectors S6 kinase 1 (S6K1), which controls ribosomal biogenesis, and eukaryotic initiation factor 4E-binding protein 1, which regulates mRNA translation. mTOR is essential to the accumulation of cell mass (growth) and increase in cell number (proliferation).[19] Rapamycin regulates the growth of endothelial cells by inhibiting mTOR complex.[18] Thus, it can be seen that hemangioma accompanied by excessive AKT activation should be particularly sensitive to rapamycin.[20]

Clinically, rapamycin is used as an immunosuppressant for solid-organ transplant recipients and in cardiovascular stents to prevent postangioplasty restenosis, but recent attention has been focused on the potential of rapamycin for treating cancer.[5–7] The Phase I and II studies with rapamycin analogs in breast and other cancers have demonstrated favorable responses.[20] Rapamycin also can target the self-renewal and vascular differentiation potential in patient-derived hemangioma stem cells.[21] Despite the potential of rapamycin for tumor treatment, its effect on hemangioma has not yet been investigated. In fact, we indeed observed that rapamycin significantly inhibited the proliferation and migration of mouse EOMA cells in vitro and vascular tumor growth in vivo. We also demonstrated that phosphaidylinositol 3-kinase/AKT/mTOR/S6 kinase signaling pathways are involved in rapamycin inhibiting the endothelial cell proliferation and migration process. AKT represents an important upstream molecule of the mTOR pathway. mTOR has 2 kinds of complexes, mTOR complex 1 (mTOR1) and mTOR complex 2 (mTOR2). AKT phosphorylation at Ser473 is activated by mTOR2 and associate with resistance to apoptosis, increased cell growth, cell proliferation, and cell energy metabolism. Studies have shown that the inhibition of mTOR1 and mTOR2 caused by rapamycin is time and cell type dependent.[22] mTOR contributes to hemangioma endothelial cell proliferation by stimulating an autocrine loop of VEGF signaling.[23,24] In our study, long-time treatment with rapamycin inhibited both mTOR1 and mTOR2 in EOMA cells. Strong inhibition of mTOR2 phosphorylation induced the hypoactivation of phospho-AKT at Ser473 after 48-hour rapamycin treatment. GSK3β, a downstream kinase of AKT, implicated in the regulation of cell proliferation and apoptosis, was downregulated here. S6, involved in the regulation of cell size and proliferation, was also inhibited significantly. So the suppression of vascular tumor results from the inhibition of both mTOR1 and mTOR2 caused by rapamycin in vascular endothelial cells. Furthermore, with the increasing importance of drug-dose effects, it is worthwhile to pursue research on low-concentration rapamycin induced suppression of hemangioma. It is already evident that rapamycin has some severe adverse reactions such as immunosuppression, hyperlipidemia, liver damage,

bone marrow suppression, and thrombocytopenia.[25] How to decrease the dose and keep the curative effect is the most critical point. The serum concentration of rapamycin in clinical application is usually within 7 to 16 nM.[26] In our study the concentration of rapamycin used in vitro was analogous to low serum concentration. In the study of in vivo 0.5 mg/kg rapamycin injected intraperitoneally in mice (about 10 g) was amount to 17.3 mg/kg relative to the dose of oral rapamycin in human beings by correcting for the route of administration, the free fraction, and the frequency of administration differences between mouse and man. It is calculated by the formula of kilogram × IP dose in mouse × coefficient of the same route of administration: (387.9) / 14% / bioavailability of IP (80%). So 0.5 mg/kg rapamycin was low dose relative to the dose of oral rapamycin (16–24 mg/kg).[27] Under the condition of low drug concentration, rapamycin also significantly inhibited the growth of hemangioma. These results should provide a low toxicity and adverse reaction alternative for the treatment of hemangioma compared with commonly used drugs like propranolol, interferons, and corticosteroids. In addition, we also tried to detect the dose-related efficacy and the safe dose range of rapamycin treatment. The mice with 2 mg/kg rapamycin injection in vivo died during the beginning days. This suggested a narrow and not very wide therapeutic index drug treatment. It encourages continued development of rapamycin and its analogs for use to increase the LD_{50} and therapeutic index in vascular tumor therapy in future research.

Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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