ORIGINAL ARTICLE

Rapamycin causes growth arrest and inhibition of invasion in human chondrosarcoma cells

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Summary

Purpose: Chondrosarcoma is a highly malignant tumor that is characterized by a potent capacity to invade locally and cause distant metastasis and notable for its lack of response to conventional chemotherapy or radiotherapy. Rapamycin, the inhibitor of mammalian target of rapamycin (mTOR), is a valuable drug with diverse clinical applications and regulates many cellular processes. However, the effects of rapamycin on cell growth and invasion of human chondrosarcoma cells are not well known.

Methods: We determined the effect of rapamycin on cell proliferation, cell cycle arrest and invasion by using MTS, flow cytometry and invasion assays in two human chondrosarcoma cell lines, SW1353 and JJ012. Cell cycle regulatory and invasion-related genes' expression analysis was performed by quantitative RT-PCR (qRT-PCR). We also evaluated the effect of rapamycin on tumor growth by us-

ing mice xenograph models.

Results: Rapamycin significantly inhibited the cell proliferation, induced cell cycle arrest and decreased the invasion ability of human chondrosarcoma cells. Meanwhile, rapamycin modulated the cell cycle regulatory and invasion-related genes' expression. Furthermore, the tumor growth of mice xenograph models with human chondrosarcoma cells was significantly inhibited by rapamycin.

Conclusions: These results provided further insight into the role of rapamycin in chondrosarcoma. Therefore, rapamycin targeted therapy may be a potential treatment strategy for chondrosarcoma.

Key words: cell cycle, cell growth, chondrosarcoma, invasion, rapamycin

Introduction

Chondrosarcoma, a type of malignant tumor originating from cartilaginous tissue, is the second most common primary malignant tumor of bone [1-3]. Chondrosarcoma is a devastating disease due to its high resistance to conventional chemotherapy and radiotherapy [4,5]. Complete surgical resection of localized disease remains the primary therapeutic modality, while tumor metastatic potential contributes to poor prognosis [5-7]. The 10-year survival rate is only 29% for grade III chondrosarcoma and there has been no progress over the last several decades [8-10]. Due to lack of an effective adjuvant therapy, there is an urgent need to identify therapeutic targets and develop novel treatment strategies for patients with this disease.

mTOR, the conserved serine/threonine kinase, is known as a crucial regulator of protein synthesis, cell-cycle progression, cell growth, proliferation, angiogenesis, apoptosis and survival [11-13]. Now, targeting mTOR as an attractive anticancer strategy has become rapidly a focus for cancer therapeutic development [13-16]. Rapamycin is a specific inhibitor of mTOR and the inhibition

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of mTOR by rapamycin is a promising anticancer therapy [17]. Rapamycin has shown specific anticancer activities in a various type of tumors, including head and neck, gastric, lymphoid malignancies and neuroblastoma [15,17,18]. Recent studies have shown that the mTOR inhibitor everolimus blocks cell proliferation and prevents *in vivo* chondrosarcoma progression [19], but so far the mechanisms underlying the mTOR inhibition have rarely been explored.

The aim of the present study was to examine whether rapamycin induced any anticancer effect on chondrosarcomas and its potential biological mechanism of action.

Methods

Cell culture and reagents

Human chondrosarcoma cell lines SW1353 and JJ012 were purchased from the American Type Culture Collection (VA, USA). Cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (Gibco, NY, USA), 2 mM glutamine and 1% penicillin/streptomycin (100 U/ml) at 37°C in a humidified incubator with 95% air and 5% CO₂.

Rapamycin (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, MO, USA) with a stock concentration of 1 mM and stored at -80° C until use. The final concentration of DMSO was 0.1%.

MTS assay

The number of viable cells was examined by using a Cell Titer-96 Aqueous One Solution Cell Proliferation Assay (MTS). 2×10^3 SW1353 and 3×10^3 JJ012 cells were seeded in 96-well plates and allowed to adhere overnight. After 48 or 72 hrs of incubation with various concentrations of rapamycin (0–25 µMoL) or 0.1% DMSO, the medium was removed and 20 µL MTS (Sigma, MO, USA) were added to each well followed by incubation for 4 hrs at 37°C. The absorbance of each well was measured at 490 nm using a microplate reader (BioRad, USA). Assays were performed in three independent experiments.

Cell cycle analysis

Cells were treated with 2 μ M rapamycin or 0.1% DMSO for 48 hrs, then were collected by trypsinization and washed with PBS for 5 min by centrifugation at 1000 rpm, followed by fixation in 70% cold ethanol at 4°C overnight. Cells were resuspended in a staining solution containing propidium iodide, Trition X-100 and RNase A and incubated for 30 min at 37°C. DNA content of the stained cell nuclei was measured by flow cytometry analysis (Becton Dickinson FACScan, CA, USA).

Invasion assay

The invasion assay was performed using 24-well cell culture chambers containing inserts with 8 μ m pores filter membranes (BD Biosciences, USA). SW1353 cells (1.5×10^4) and JJ012 cells (1.0×10^4) were seeded in serum-free media and incubated for 24 hrs at 37 °C in 5% CO₂. Cells that invaded through the filter onto the underside of the membranes were fixed in 3.7% formal-dehyde for 5 min and stained with 0.1% crystal violet for 15 min. Invaded cells were quantitated under a microscope.

Quantitative real-time PCR

Total RNA was extracted from chondrosarcoma cells after treatment with rapamycin (2 μ MoL) for 48 hrs using a TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The purificated RNA was converted to complementary DNA using oligo-dT primer and Superscript III Reverse Transcriptase (Gibco, NY, USA). The qRT-PCR analysis was performed using SYBRR Premix Ex Taq (Takara, Japan). GAPDH was used as the internal control to normalize the target mRNAs' expression. The primers used for amplification of genes were as follows: cdk2, 5'-CCAGGAGTTACTTCTATGCCTGA-3'(sense) and 5'-TTCATCCAGGGGAGGTACAAC-3'(antisense); cdk4, 5'-AT-GGCTACCTCTCGATATGAGC-3'(sense) and 5'-CATTGG-GGACTCTCACACTCT-3'(antisense); cyclin D1, 5'-GCTG-5'-CCTCCT CGAAGTGGAAACCATC-3'(sense) and TCTGCACACATTTGAA-3'(antisense); epidermal growth factor (egf), 5'-TGGATGTGCTTGATAAGCGG-3'(sense) and 5'-ACCATGTCCTTTCCAGTGTGT-3'(antisense); basic fibroblast growth factor 2 (fgf2), 5'-AGAAGAGCGACCCT-CACATCA-3'(sense) and 5'-CGGTTAGCACACACTCCT-TTG-3'(antisense); matrix metalloproteinase-2 (MMP-2), 5'-AATGCCATCCCCGATAACC-3'(sense) and 5'-GCTCAG-CAGCCTAGCCAGTC-3'(antisense); GAPDH, 5'-GTCT-TCACCACCATGGAGAACA-3'(sense) and 5'-ATCCA-CAGTCTTCTGGGTGG -3'(antisense).

Animal studies

The animal experimentation was approved by the Institutional Animal Care and Use Committee at Shandong Jining No.1 People's Hospital. SW1353 cells (1.2×10^7) and JJ012 cells (1×10^6) were washed and resuspended in PBS and subcutaneously injected into the right flank of the eight-week-old nude female mice. Once the cells developed palpable tumors with an average size of approximately 100 mm³, the mice were randomly divided into two groups for daily injection of rapamycin (80 mg/kg) or 0.1% DMSO (vehicle control) for 4 weeks. Mice were observed daily and tumor volumes and body weights were measured twice a week. Tumor volumes were calculated with the formula: length×width²/2.

Statistics

Values of all measurements were expressed as

mean \pm standard error of the mean (SEM). Statistical comparison was performed using the Student's t-test and a p value < 0.05 was considered to indicate statistically significant differences.

Results

Rapamycin inhibits the proliferation of human chondrosarcoma cells

To assess the role of rapamycin in chondrosarcoma cell proliferation, we incubated the SW1353 and JJ012 cells with various concentrations of rapamycin (0, 0.008, 0.04, 0.2, 1.0, 5.0, 25 µM) for 48 and 72 hrs and the cell proliferation of chondrosarcoma cells was measured by MTT assay. As shown in Figure 1a, rapamycin treatment resulted in significant inhibition of cellular proliferation rate of SW1353 cells over the period of both 48 and 72 hrs compared to the untreated control cells. Similarly, rapamycin treatment both 48 and 72 hrs decreased the proliferation rate of JJ012 cells (Figure 1b). These data suggested that rapamycin may play an essential role in the proliferation of chondrosarcoma cells.

Effects of rapamycin on the cell cycle of chondrosarcoma cells

The inhibitory effect of rapamycin on cell proliferation of chondrosarcoma cells could be a result of cell cycle arrest. Therefore, flow cytometry was carried out to examine whether rapamycin altered the distribution of chondrosarcoma cells in different phases of the cell cycle. Compared with vehicle treated cells, rapamycin-treated SW1353 cells exhibited a significant accumulation of cells in the G1 phase with a corresponding reduction of cells in the S and G2/M phases (Figure 1c). We also found that the treatment of JJ012 cells with rapamycin led to a substantial increase in the proportion of cells in the G1 phase, associated with a concomitantly decreased proportion of cells in the S and G2/M phases, compared with cells treated with vehicle (Figure 1d). These results indicated that rapamycin induced G1 arrest and thus explained the drastic reduction in the proliferation rates of the chondrosarcoma cells observed.

Effects of rapamycin on the invasion ability of chondrosarcoma cells



Figure 1. Inhibitory effects of rapamycin on the cell proliferation and cell cycle of chondrosarcoma cells. Rapamycin significantly inhibited the cell proliferation of SW1353 (a) and JJ012 (b) cells both at 48 and 72 hrs. Rapamycin induced G1 arrest in SW1353 (c) and JJ012 (d) cells. Data are presented as mean ± SEM, * p<0.05, compared with vehicle control group.

To evaluate whether rapamycin contributes to tumor invasive potential, invasion assay was applied to determine the role of rapamycin in cell invasion of chondrosarcoma cells. As shown in Figure 2a, compared to vehicle control group, rapamycin markedly reduced the cellular invasion in SW1353 cells. Results also showed that the percentage of invasion was drastically reduced in rapamycin-treated JJ012 cells (Figure 2b). These results indicated that rapamycin is important for cellular invasion *in vitro* and hence may con-tribute to the invasive potential of chondrosarcoma.

Effects of rapamycin on the expression of cell cycle regulatory and invasion-related genes

In order to elucidate the mechanism of rapamycin in the inhibition of chondrosarcoma cell cycle progression, we examined the effect of rapamycin on the genes' expression levels of CDK2, CDK4 and cyclin D1, the critical factors for G1 checkpoint. As shown in Figure 3a, the expression of CDK2, CDK4 and cyclin D1 was dramatically decreased in SW1353 cells by rapamycin treatment. Rapamycin downregulated the expression of CDK2 and CDK4 with no significant effect on cyclin D1 expression in JJ012 cells (Figure 3b). These results suggested that rapamycin modulated the cell cycle regulatory genes' expression, by which it could induce cell cycle arrest of chondrosarcoma cells.

To investigate the mechanism by which

rapamycin causes decreased invasion ability of chondrosarcoma cells, we measured the effects of rapamycin on the expression of invasion-related genes, epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and MMP-2. We found that the genes' expression of EGF and MMP-2 were significantly inhibited with no effect on cyclin D1 by rapamycin treatment in SW1353 cells (Figure 3c). In addition, rapamycin treatment significantly inhibited all the three genes' expression (Figure 3d). These results indicated that rapamycin suppressed chondrosarcoma cell invasion by regulating the expression of invasion-related gene.

Antitumor acitivity of rapamycin in osteosarcoma xenografts

On the basis of rapamycin-induced antiproliferative effect exhibited *in vitro*, we carried on to detect whether rapamycin possessed also antitumor activities *in vivo*. Therefore, mice xenograft models of SW1353 and JJ012 cell lines were established and mice were treated with rapamycin or vehicle daily for 28 days. As shown in Figure 4a, the tumor volume in mice bearing SW1353 cell treated with rapamycin was markedly decreased compared with the vehicle control group. Meanwhile, rapamycin significantly suppressed the tumor growth of JJ012 cells. No significant weight loss of the mice was observed (Figure 4b). These results provided further confirmation of the antitumor effects of rapamycin on chondrosarco-



Figure 2. Rapamycin inhibits the invasion ability of human chondrosarcoma cells. Rapamycin treatment resulted in a qualitative decrease in the number of invading SW1353 (a) and JJ012 (b) cells. The invading cells were visualized by microscopy (x100). Data are presented as mean \pm SEM. * p<0.05, compared with vehicle-treated chondrosarcoma cells.



Figure 3. Effects of rapamycin on the expression of cell cycle regulatory and invasion-related genes. Rapamycin modulated the expression of cell cycle regulatory genes in SW1353 (**a**) and JJ012 (**b**) cells. Rapamycin modulated the expression of invasion-related genes in SW1353 (**c**) and JJ012 (**d**) cells. Data are presented as mean ± SEM. * p<0.05, compared with vehicle control group.



Figure 4. Inhibition of tumor growth by rapamycin in nude mice xenografted with chondrosarcoma cells. Rapamycin (80 mg/kg) significantly inhibited the tumor growth of SW1353 (a) and JJ012 (b) cells. Tumor volumes are presented as mean \pm SEM. * p<0.05, # p<0.01, compared with vehicle control group.

ma cell growth in vivo.

Discussion

With the advent of systemic chemotherapy, the long-term survival has been increased in patients with mesenchymal malignancies such as osteosarcoma and Ewing's sarcoma [20,21]. However, as the second most common primary bone tumor and the second highest cause of cancer-related mortality in the pediatric age group, chondrosarcomas are notorious for their lack of response to systemic chemotherapy, poor prognosis and survival [6,10]. Therefore, it is important to create an effective adjuvant therapy for preventing chondrosarcoma growth and improving patient survival. In the present study we described the effect of the specific mTOR inhibitor, rapamycin, on the chondrosarcoma cell growth, cell cycle distribution and cell invasion and tried to elucidate the mechanism underlying the cell proliferation, cell cycle arrest and invasion.

First, we explored the growth-inhibitory activity of rapamycin and MTT assay was used to evaluate the effects of rapamycin on chondrosarcoma cells. Our results showed that rapamycin was effective in inhibiting the proliferation of SW1353 and JJ012 cells. As has been shown previously, blockade of cells in G1 phase by rapamycin was found in several types of cancers, such as neuroblastoma, osteosarcoma, lymphoid and renal tumors [18,22-24]. Given these similarities, we proposed that the growth suppression of chondrosarcoma cells by rapamycin in vitro may rely on cell cycle blockade and flow cytometry assay was used to investigate the cell cycle distribution. We showed a substantial increase in the proportion of SW1353 and JJ012 chondrosarcoma cells in the G1 phase of the cell cycle after treatment with rapamycin. The results suggested that rapamycin exerts growth inhibitory effects on chondrosarcoma cells by leading to cell cycle arrest at G1 phase, and thus not allowing the cells to progress further in the cell cycle and proliferation.

To evaluate the molecular basis underlying cell cycle arrest, cell cycle regulatory genes were probed. As it is known, cell cycle progression could be positively regulated by cyclin-dependent kinases and cyclin D1 [25]. We found that rapamycin significantly decreased the expression of CDK2, CDK4 and cyclin D1 in SW1353 cells and downregulated CDK2 and CDK4 expression in JJ012 cells. Therefore, we claim that the downregulation of cell cycle regulatory genes' expression contributed to the blockade of cell cycle arrest in G1 phase induced by rapamycin in chondrosarcoma cells lines.

We next determined the effects of rapamycin on the cell invasion ability of chondrosarcoma cells by using invasion assay. Our results showed that rapamycin treatment resulted in significantly decreased level of invasion in SW1353 and JJ012 cells. We presumed that rapamycin may affect the level of invasion-related genes, such as MMP-2, EGF and FGF2, so qRT-PCR was performed. Previous studies demonstrated that MMP-2 is linked to tumor invasion and metastasis as well as to poor prognosis [26-28]. Other studies indicated that EGF and FGF2 act as essential regulators of cell proliferation, tumor angiogenesis and invasion [29-33]. qRT-PCR results revealed that rapamycin treatment significantly downregulated the mRNA expression levels of EGF, FGF2 and MMP-2 in JJ012 cells, with no significant effect on FGF2 expression in SW1353 cells. Our results speculated that the inhibitory effect of rapamycin on chondrosarcoma cell invasion may be based on the downregulation of invasion-related genes' expression.

Finally, we focused on the role of rapamycin in chondrosarcoma cells *in vivo*. We found that rapamycin significantly suppressed tumor growth in a xenograft mouse model of chondrosarcoma cells without obvious bodyweight loss. These results further confirmed the *in vitro* inhibitory effect of rapamycin on the proliferation of chondrosarcoma cells.

The results presented in this report revealed that rapamycin inhibited the proliferation of chondrosarcoma cells *in vitro* and *in vivo* and reduced the invasive capacity of chondrosarcoma cells *in vitro*, a fact that may help increase the understanding of the mechanism underlying chondrosarcoma growth and invasion. In conclusion, this study demonstrated that rapamycin has a crucial role in suppressing growth and invasion of chondrosarcoma and may be a potential therapeutic target for the treatment of human chondrosarcoma.

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