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Rapamycin inhibits cell growth by induction of apoptosis on hepatocellular carcinoma cells in vitro

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Abstract

Background: Rapamycin, isolated from *Streptomyces hygroscopicus*, is recently reported to have immunosuppressant and anti-tumor effects on a large variety of cancers. To date, no detailed data are available about the effects of rapamycin on hepatocellular carcinoma cells.
Objective: In this study, the anti-proliferation effects of rapamycin on hepatocellular carcinoma cells BEL-7402 and HepG-2 in vitro were studied.
Methods: Cell viability was assessed by MTT assay and [^{3}H]-thymidine uptake, cell apoptosis was observed by Hoechst 33258 staining and flow cytometry (FCM). The variation of caspase-3 and apoptotic related genes was assayed by Western blotting, cell mitochondrial membrane potential was also investigated by using standard methods.
Results: Rapamycin could inhibit the growth of hepatocellular carcinoma cells and cause apoptosis significantly; the suppression was both in time- and dose-dependent manner, marked morphological changes of cell apoptosis were observed very clearly by Hoechst 33258 staining. Rapamycin exhibits induction apoptosis by activation of caspase-3 and disruption of the mitochondrial membrane potential on hepatocellular carcinoma cells in vitro. Western blotting analysis demonstrated that anti-apoptotic protein Bcl-2 was down-regulated while pro-apoptotic protein Bcl-xl up-regulated remarkably in a time-dependent manner when apoptosis occurred.
Conclusion: Rapamycin has significant anti-proliferation effect by induction of apoptosis via activation of caspase-3 and disruption of mitochondrial membrane potential, as well as by down-regulation of anti-apoptotic protein Bcl-2 and up-regulation of pro-apoptotic protein Bcl-xl on hepatocellular carcinoma cells. The data provide a potential mechanism for rapamycin-induced apoptosis in hepatocellular carcinoma cells, suggesting that rapamycin may serve as both an effective adjunctive reagent for the treatment of residual cancer cells and immunosuppressant after liver transplantation of hepatocellular carcinoma, and that in vivo anti-cancer effects as well as its potential clinical effectiveness need further investigation.
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Keywords: Rapamycin; Hepatocellular carcinoma; Caspase-3; Mitochondrial membrane potential; Bcl-2

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant diseases and has become increasingly important all over the world. HCC is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death [1]. Therefore, it is a permanent subject to find new methods for the treatment of HCC. There are many methods in the treatment of HCC such as chemotherapy by using new anti-tumor drugs, operation, intervene therapy, liver transplantation, and so on. Among these methods, liver transplantation has now been considered one of the most curative treatment options for HCC. It is reported that the current 1- and 5-year survival rates for HCC patients undergoing orthotopic liver transplantation are 77.0% and 61.1%, respectively, and the 5-year survival rate has steadily improved from 25.3% in 1987 to 61.1% during the most recent period studied [2]. Though liver transplantation is an effective method nowadays in the treatment of HCC, there is still a certain recurrence after liver transplantation [3].

Rapamycin, a derivative isolated from *Streptomyces hygroscopicus*, is recently reported to have immunosuppressant and anti-tumor effects on a large variety of cancers, and recent data have shown that it is a new therapeutic strategy to use rapamycin

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as a single agent as well as in combination with other chemicals in the treatment of both primary and metastatic tumor cells [4].

Though rapamycin has been proved to have anti-tumor effects in many different human cancer cells, many of its anti-proliferation and apoptotic mechanisms remain to be demonstrated. To date, no detailed data are available about the role and mechanisms of rapamycin in hepatocellular carcinoma cells. In order to understand the roles of rapamycin in hepatocellular carcinoma cells and possible clinical application of rapamycin in hepatocellular carcinoma therapy, we examined the anti-proliferation effects of rapamycin on hepatocellular carcinoma cells in vitro.

2. Materials and methods

2.1. Main reagents

Rapamycin isolated from *S. hygroscopicus* was obtained from Sigma-Aldrich (St. Louis, MO). A carrier solution was produced by using a diluent containing Tween, *N-N*-dimethylacetamide, and polyethylene glycol 400 (from Sigma-Aldrich). Hoechst 33258 was purchased from Sigma Company. The antibodies used in this study, anti-Bcl-2, anti-Bcl-xl, and anti-Bax, were purchased from Santa Cruz Company (Germany). Antibodies against caspase-3 were bought from Upstate Inc. Caspase inhibitor (z-DEVD-FMK) was purchased from R&D systems Inc (Minneapolis, MN, US). TRIZOL was from GIBCO (USA), and caspase colorimetric assay kit was from MBI (USA).

2.2. Cell culture

Hepatocellular carcinoma cells BEL-7402 and HepG-2 were provided by central laboratory of Sun Yat-sen University Cancer Center. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS), 100 U/mL penicillin and l00 μg/mL streptomycin, in a humidified incubator with 5% CO_2 at 37 °C. All the cells were passaged twice weekly and routinely examined for mycoplasma contamination. Cells in logarithmic growth phase were used for further experiments.

2.3. Cell viability assay

The viability of the cells was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, hepatocellular carcinoma cells in logarithmic growth phase were collected, and 2×10^5 cells/well were dispensed within 96-well culture plates in 100 mL volumes. Then different concentrations of rapamycin (5 nmol/L, 10 nmol/L, 20 nmol/L, 30 nmol/L, 40 nmol/L and 50 nmol/L) were put in different wells. Each of the concentrations above was regarded as one treated group while there was no rapamycin in the control group. Each of the treated or control group contained 6 parallel wells. Before MTT assay, the cells were first incubated in serum free RPMI for 24 h. After rapamycin was absorbed completely, culture plates were then maintained in RPMI containing 10% fetal calf serum for 0, 24, 48 and 72 h prior to the addition of tetrazolium reagent. MTT working solution was prepared as follows: 5 mg MTT/mL PBS was sterile by being filtered with 0.45 μm filter units. Each of the above cultured wells was added 20 μL of MTT working solution and then incubated continuously for 4 h. The water insoluble formazan was formed during incubation and it was solubilized by adding solubilization agent to each well. Amount of formazan was determined by measuring the absorbance at 540 nm using an ELISA plate reader.

2.4. [^{3}H]-thymidine uptake

For cell proliferation assay, [^{3}H]-thymidine uptake was used to examine the effect of rapamycin on cell growth. Briefly, 1×10^5 per mL cells were treated with various concentrations of rapamycin in 96-well plates. After 0, 24, 48, 60 and 72 h of incubation, 1 μCi [^{3}H]-thymidine (Beijing Atomic Energy Research Institute, Beijing, China)/well was added to the 96-well plates, the plates were incubated for an additional 12 h at 37 °C, and the cells were harvested and counted for incorporated radioactivity.

2.5. Apoptosis detection by flow cytometry (FCM)

For FCM analysis, BEL-7402 and HepG-2 cells treated with different concentrations of rapamycin for different times were collected, pelleted, washed with PBS, and resuspended in PBS containing 20 mg/L PI and 1 g/L ribonuclease A. 1×10^6 fixed cells were examined per experimental condition by flow cytometry. The percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-G_1) DNA divided by the total number of cells examined.

2.6. Hoechst 33258 staining

The morphology of BEL-7402 and HepG-2 cells exposed to rapamycin for different time was observed firstly under inverted microscope. Then Hoechst 33258 staining was used to observe the apoptotic morphology. Cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min, stained by Hoechst 33258 (10 mg/L) for 1 h, and then subjected to fluorescence microscopy. After treatment with rapamycin, the morphologic changes including reduction in the volume and nuclear chromatin condensation were observed.

2.7. Western blot analysis

For Western blotting, cells were washed with ice-cold PBS twice and lysed for 30 min at 4 °C, then debris was removed by centrifugation for 15 min at 15,000 ×*g* at 4 °C, and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose filters. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filters were reacted firstly with the primary antibodies at 4 °C overnight, followed by extensive washes with PBS twice and TBST twice. Filters were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, washed with TBST and developed using the Super Signal West Pico Kit.

2.8. Analysis of the mitochondrial membrane potential ($\Delta\psi m$)

The mitochondrial membrane potential ($\Delta\psi$m) was measured by FCM using the intramitochondrial dye JC-1(Alexis Biochemical Co, Germany) after BEL-7402 and HepG-2 cells treated with rapamycin for 48 h. The detection procedure was performed according to the manufacture's instructions. Data were converted to dot plots using Cell Quest software (Becton Dickison, Germany).

2.9. Caspase activity assay

The activity of caspases was determined by caspase colorimetric assay kit, according to the manufacturer's protocol. Briefly, rapamycin treated cells were washed with ice-cold PBS and lysed in a lysis buffer. The cell lysates were tested for protease activity using a caspase-specific peptide, conjugated to the color reporter molecule *p*-nitroanaline. The chromophore *p*-nitroanaline, cleaved by caspases, was quantitated with a spectrophotometer at a wavelength of 405 nm. The caspase enzymatic activities in cell lysate are directly proportional to the color reaction.

2.10. Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed with a Student's *t*-test using SAS 6.12 software. Statistical significance was accepted at the level of $p < 0.05$.

3. Results

3.1. Cell growth inhibition effects of rapamycin on hepatocellular carcinoma cells

To investigate the cytotoxicity of rapamycin on hepatocellular carcinoma cells, BEL-7402 and HepG-2 cells were treated with various concentrations of rapamycin for 0, 24, 48 and 72 h. As shown in Fig. 1, rapamycin (over 10 nmol/L) had significant growth inhibition effects

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