Effects of rapamycin on prostate cancer PC-3 cells

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[Abstract] Background and Objective: The mammalian target of rapamycin (mTOR) signaling network regulates cell growth, proliferation, survival and apoptosis. This study was to investigate the effect and the underlying mechanism of rapamycin on prostate cancer PC-3 cells. Methods: PC-3 cells were treated with 1 mmol/L rapamycin. The proliferation of PC-3 was examined by MTT. The cell cycle distribution of PC-3 was measured by FCM. The protein levels of raptor, rictor, Akt, pS6k1-T389, pAkt-s473 in PC-3 were examined by western blot. Results: Rapamycin increased the proliferation of PC-3 at 24 h, however, it remarkably inhibited cell proliferation after 36 h (P<0.01), which became more obviously at 72 h. Although incubation with rapamycin slightly induced cell arrest at the S phase at 24 h, this gradually increased PC-3 cells at the G1 phase at 36 h and 48 h. Compared with the control group, the protein levels of raptor and pS6k1-T389 were significantly decreased (P<0.01), and those of rictor and Akt remained unchanged after the treatment with rapamycin for 24 h; the protein level of pAkt-s473 was significantly increased at 24 h (P<0.01), but was obviously inhibited at 36 h and almost completely inhibited at 72 h (P<0.01). Conclusions: Prolonged rapamycin treatment inhibits the proliferation of PC-3 cells. This may be caused by rapamycin-induced cell cycle arrest at the G1 phase and inhibition of Akt phosphorylation.

Key word: rapamycin, Akt, PC-3, mTOR, cell cycle, prostate cancer

Prostate cancer is one of the main diseases threatening men's health. In West countries, the incidence of prostate cancer ranks the highest in male cancers and the mortality rate is the second highest following lung cancer.1 In China, prostate cancer has gradually become a common malignant disease in men. Its incidence rate has increased significantly with the prolonged average age, changes in diet, increased intake of high-fat food.2 The regular hormonal therapy has no obvious effect on androgen-independent prostate cancer. At present, the mechanism of the shift of androgen-dependent to androgen-independent prostate cancer cells and continuous proliferation of cells without androgen has not been fully elucidated.

mTOR is a downstream molecule of the phosphatidylinositol 3-kinase / protein kinase B (PI3K/Akt) signaling pathway. mTOR
participates in and plays a central role in the regulation of varied biological functions like gene transcription, protein translation, ribosomal biosynthesis, cell apoptosis and so on, particularly in proliferation and invasion of cancer cells. Akt can promote cell growth, proliferation and survival. In many cancer cells, Akt is highly activated. The anti-cancer effect of rapamycin on parts of cancer cells has been gradually realized. So it is necessary to elucidate the mechanism of the effect of rapamycin on cancer cells and its signaling pathways. This study was to investigate the effect and the underlying mechanism of rapamycin on prostate cancer PC-3 cells.

Materials and Methods

Main materials and instruments. Prostate cancer cell line PC-3 was stored in our laboratory. The following reagents were used: DMEM culture medium (Gibco BRL, USA), fetal calf serum (FCS) (Hangzhou, Sijiqing bioengineering Co. Ltd, P.R. China), MTT (Sigma, USA), rapamycin and polyclonal antibody raptor, rictor, Akt, pAkt-s473, pS6K1-T389 (cell signaling), propidium iodide (Caltag, USA), ELISA reader (Bio-Rad), flow cytometer of EPICS XL-4 type (Beckman coulter).

Methods

Cell culture. PC-3 cells were cultured in DMEM medium containing 10% FCS, 100u/mL penicillin and 100u/mL streptomycin at 37°C in a humidified incubator containing 5% CO2. The cells were digested by 0.25% trypsin to passage the culture. Cells at the logarithmic phase of growth were used for further experiments.

MTT assay. PC-3 cells were digested with 0.25% trypsin to prepare single cell suspension. Then 200 µl cell suspension was seeded into a 96-well plate at a density of 5 × 10⁴ cells/well. After adherence, the cells were treated with 1nmol/L rapamycin. Cells in the negative control group were added with an equal volume of medium. Cells were incubated at 37°C, 5% CO₂ for 24h, 36h, 48h, and 72h. MTT (5 mg/ml) 20ul was added to each well and incubated at 37°C, 5% CO₂. After 4 h, cultivation was terminated and the medium was discarded. DMSO (150 ul) was added into each well and the plate was vibrated for 10 min to dissolve crystal. The absorption (A) was measured at 490 nm (A₄₉₀). The blank well was used for zero setting. The experiment was repeated for three times. The cell inhibition rate = (A₄₉₀ value of the negative control group - A₄₉₀ value of the treatment group) / A₄₉₀ value of the negative control group × 100%

Detection of cell cycle distribution. PC-3 cells during logarithmic phase of growth were digested with 0.25% trypsin to prepare single cell suspension. Then cell suspension was seeded into new culture bottles at a density of 5 × 10⁴ cells per bottle. After adherence of cells, the medium of the treatment groups was changed with fresh medium containing 10% inactivated FCS and 1nmol/L rapamycin; the negative control group was changed with an equal volume of fresh medium. Each drug dose group had three doubled wells. The blank control group was added with medium only. Cells were collected at 24h, 36h, 48h, and 72h, respectively, and fixed with 70% cold ethanol, 4°C overnight. Before detection, cells were washed with PBS, the supernatant was centrifugated and discarded, 1ml of 50ug/ml RNase was added, and incubated at water bath at 37°C for 30 min. The supernatant was centrifugated and discarded. After adding 1ml of 60ug/ml PI, cells were incubated for 30 min away from light, filtered by the nylon net with 180 screen opening, detected by flow cytometry.

Western blot. Cells were routinely collected, washed with PBS for three times. Cell lysis buffer was added to extract the total protein. After determination of the protein density by the BCA method, 40 ug protein samples were used for SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes, incubated with 5% BSA at room temperature for 2 h, then incubated respectively with anti-rictor (1:2000), anti-raptor (1:250), anti-Akt (1:1000), anti-pAkt-Ser473 (1:1000) and anti-pS6K1-T389 (1:1000) at 4°C overnight, washed with TBST for 10 min for three times, incubated with
secondary-antibodies tagged by horseradish peroxidase for 1 h, washed for three times, incubated with freshly prepared illuminant liquid for 3 min, exposed to X-ray films in dark room and visualized.

Statistical analysis. All data are expressed as mean ± SD. Paired comparison was performed using students t-test. P<0.05 was considered statistically significant. All data were performed using SPSS 10.0 software package.

Results

Effect of rapamycin on proliferation of PC-3 cells. Compared with the control group, rapamycin increased the proliferation of PC-3 cells at 24h, however, it remarkably inhibited proliferation of PC-3 cells at 36h and 48h (p<0.01), which became more obviously at 72h (p<0.01): compared with other experiment groups, rapamycin remarkably inhibited proliferation of PC-3 cells at 72h (p<0.01) (Fig.1).

Effect of rapamycin on cell cycle distribution of PC-3 cells. Compared with the control group, cells in S phase were increased to some extent after 24 h treatment with rapamycin, however, PC-3 cells in G1 phase were increased gradually after 36 h, 48 h and 72h (Fig. 2). As shown in Fig.2, with the prolonged treatment with rapamycin, PC-3 cells were mainly blocked at G1 phase (p<0.01).

Effect of rapamycin on raptor, rictor, Akt, pAkt-s473, pS6k1-T389 proteins in PC-3 cells. The expression of raptor was significantly decreased after 24 h treatment with rapamycin (p<0.01), but rictor and Akt were barely altered; the expression of pAkt-s473 was significantly increased at 24 h, but was obviously inhibited at 36 h and almost completely inhibited at 72 h (p<0.001); the expression of pS6k1-T389 was significantly decreased after the treatment with rapamycin for 24 h (Fig.2, Table 3).
Discussion

Recently mTOR has been found to have two different types of functional complexes, mTORC1 and mTORC2. mTORC1 contains mTOR, mLST8 and regulatory-associated protein of mTOR (raptor). The activation of mTOR1 may activate ribosome S6K1. The complex of rapamycin and FKBP12 proteins can bind to the FRB structural domain of the mTOR, destroy mTOR-raptor interaction, make the mTOR kinase domain to lose its ability to approach and phosphorylate downstream substrates, resulting in inhibiting the activity of the mTOR-raptor complex activity, and making mTORC1 to be sensitive to rapamycin. mTORC2 contains mTOR, mLST8, sin1, PRR5 and rapamycin-insensitive companion of mTOR (rictor). mTORC2 regulates Akt-Ser473 phosphorylation. The FKBP12-rapamycin complex can not bind to mTOR-rictor, thus the mTOR-rictor complex is not sensitive to rapamycin. A recent study showed that prolonged rapamycin treatment can inhibit the assembly of mTORC2.

The anti-proliferation effect of rapamycin and its derivative targeting mTOR on cancer cells has been already proved in clinic. The derivative, CCI-779, inhibits the mTOR signaling pathway by combining with FKBP12, suppresses tumor growth by regulating cell cycle distribution and angiogenesis. In a large scale of clinical trials, the total survival time of renal cell carcinoma patients treated with CCI-779 alone was obviously longer than those treated with IFN alone; CCI-779 has shown a remarkable advantage to be used as a molecular target drug to treat metastatic renal cell carcinoma.

In the prophase, prostate cancer patients are sensitive to rapamycin treatment, but most of them would relapse quickly after temporary regression and further progress to androgen-independent prostate cancer with strong invasive capacity. So far, there is no effective treatment for this kind of disease. In this research, androgen-independent prostate cancer PC-3 cells were treated with rapamycin. Cells proliferated at 24 h instead of being inhibited. However, the inhibition ratio was significantly increased over the time, especially at 72 h. FCM indicated that, cells in S phase were increased to some extent after 24 h treatment with rapamycin, however, after 36 h, 48 h and 72h treatment, cells in G1 phase were increased and proliferation was gradually inhibited. To explain this phenomenon, expressions of raptor, rictor, Akt, pAkt-s473 and pS6k1-T389 were detected. The protein levels of raptor and pS6k1-T389 were significantly decreased, and those of rictor and Akt were almost unchanged after the treatment with rapamycin for 24 h. The expression of pAkt-s473 was increased at 24 h, but was inhibited at 36 h and obviously inhibited at 72 h. It is suggested that activated Akt plays a main role in cell proliferation, differentiation, migration and vascularization in androgen-independent prostate cancer, through phosphorylating a series of downstream target proteins. Among the highly malignant prostate cancers, activation of Akt increases the drug resistance of cancer cells against chemotherapeutics. Akt also regulates cell cycle through many ways: (1) phosphorylation of p21 and p27kip1 to make them to accumulate in cells, and prevent them from binding to CDKs and proliferating cell nuclear antigen, thus to accelerate the progress of cell cycle, promote cells from G1 phase into S phase; (2) down-regulation of the transcription factor AFX of the forkhead family through phosphorylation to decrease the transcription of p27kip1; (3) an increase in the transcription and translation of


