Original Article

Effect of autophagy on the shikonin induced apoptosis of human medullary thyroid carcinoma TT cells

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Abstract: Objective: This study aimed to investigate the role of autophagy in the shikonin (SKI) induced apoptosis in human medullary thyroid carcinoma TT cells and the potential molecular mechanism. Methods: Human medullary thyroid carcinoma TT cells were used in the present experiment. The proliferative capability was evaluated by MTT assay, the cell apoptosis rate was determined by flow cytometry, the ultrastructure of cells was observed by transmission electron microscopy, and Western blot assay was performed to examine the expression of autophagy proteins (LC3 and p62/SQSTM1) and apoptosis-related proteins (cleaved caspase3 and cleaved PARP). Results: As compared to blank control group, SKI was able to inhibit the growth of TT cells in a dose dependent manner; after treatment with 2 and 4 μg/mL SKI for 24 h, the apoptosis rate increased significantly (24.3%±6.3% and 50.2%±8.8%, respectively, P<0.05 vs 6.7%±3.2% in control group), the expression of cleaved caspase3 and cleaved PARP increased markedly; typical vacuoles in apoptosis were observed in SKI treated cells, and the LC3-II expression increased, but p62/SQSTM1 expression decreased; in the presence of autophagy inhibitor chloroquine, SKI significantly increased the apoptosis rate and the expression of cleaved caspase3 and cleaved PARP as compared to cells treated with SKI alone. Conclusion: SKI was able to induce the autophagy and apoptosis of TT cells, and to inhibit the autophagy of TT cells may accelerate their apoptosis, suggesting that protective autophagy may partially antagonize the SKI induced inhibition of TT cell proliferation.

Keywords: Shikonin, medullary thyroid carcinoma, TT cells, autophagy, apoptosis

Introduction

Medullary thyroid carcinoma (MTC) originates from the parafollicular cells in the thyroid and accounts for about 5%-10% of thyroid malignancies [1]. The parafollicular cells of the thyroid can not absorb iodine and are insensitive to radiation. Thus, surgery and pharmacotherapy to directly kill cancer cells are the major therapeutic strategies for MTC. In recent years, natural medicines have been confirmed to have anti-tumor effects (such as paclitaxel, matrine and oridonin) [2]. Thus, to screen active ingredients or effective parts from available Chinese herbs may become a feasible way in the therapy of MTC.

Shikonin (SKI) is a small molecules naphthoquinone compound extracted from the natural medicinal plant Lithospermum erythrorhizon [3]. Studies have confirmed that SKI and its derivatives have anti-oxidative, anti-inflammatory, anti-viral and anti-tumor activities. SKI alone or in combination with other chemotherapeutics may inhibit the proliferation of cancer cells such as human promyelocytic leukemia cells, liver cancer cells and breast cancer via regulating their proliferation, metabolism, differentiation and signal transduction [4-7]. In the present study, the autophagy and apoptosis of MTC TT cells were investigated following SKI treatment in vitro, and the expression of autophagy and apoptosis related proteins. Furthermore, the autophagy inhibitor chloroquine and SKI were used to treat TT cells, aiming to investigate the role of autophagy in the SKI induced proliferation inhibition of TT cells.

Materials and methods

Drugs and cell lines

SKI was purchased from the MUST Biological Technology Co., Ltd. in Chengdu (purity: ≥98%).
Table 1. Effect of SKI with various concentrations on proliferation of TT cells (x±s, n = 3)

<table>
<thead>
<tr>
<th>Group (μg/mL)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
</tr>
<tr>
<td>1</td>
<td>81.1±6.1</td>
</tr>
<tr>
<td>2</td>
<td>70.6±4.7*</td>
</tr>
<tr>
<td>4</td>
<td>48.6±4.8**</td>
</tr>
<tr>
<td>6</td>
<td>27.5±2.8**</td>
</tr>
</tbody>
</table>

Notes: *P<0.05, **P<0.01 vs control group.

SKI was dissolved in DMSO at a density of 50 mg/mL. The SKI at a target concentration was prepared with this SKI solution. Human MTC TT cells were purchased from the Cell Bank of Chinese Academy of Science.

Reagent and instrument

Fetal bovine serum (FBS), F12-K medium, 0.25% trypsin-0.02% EDTA (Thermo), 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide (MTT), chloroquine (CQ) (Sigma), Annexin V-FITC apoptosis assay kit (BD Pharmingen), rabbit anti-human LC3, p62/SQSTM1, cleaved-caspase3, cleaved-PARP and GAPDH polyclonal antibodies, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology), and ECL chemiluminescence kit (Thermo) were used in the present study. Cell incubator (Thermo), iMARK microplate reader (Bio-Rad), Canto II flow cytometry (BD), transmission electron microscope (ZEISS), electrophoresis system (BIO-RAD) and gel imaging system (BIO-RAD) were used in the present study.

Evaluation of cytotoxicity of SKI by MTT assay

Human MTC TT cells were maintained in complete F12-K medium containing 10% FBS in a humidified environment with 5% CO₂ at 37°C. TT cells were seeded into 96-well plates at a density of 5×10⁵ cells/well. When the cells were adherent to the wall, the supernatant was removed, and SKI (100 μL) was added to TT cells at different concentrations (1, 2, 4 and 6 μg/mL) (three wells at each concentration). In blank control group, medium of equal volume was added. After treatment for 24, 48 and 72 h, MTT assay was done for the detection of cell proliferation by measurement of absorbance (A) at 490 nm in a microplate reader. The cell viability was calculated as follow: viability = (A_division/A_blank_control) ×100%. Experiment was done three times.

Detection of apoptosis rate by flow cytometry

TT cells were seeded into 96-well plates at a density of 3×10⁵ cells/well. When the cells were adherent to the wall, the supernatant was removed, and cells were incubated with SKI at 2 and 4 μg/mL. Cells in blank control group were maintained in medium of equal volume. In addition, the influence of autophagy on the cell apoptosis was investigated in blank control group, 4 μg/mL SKI group, 5 μmol/L CQ group, 4 μg/mL SKI+5 μmol/L CQ group. After 24-h treatment, cells in different groups were harvested and suspended in 1× binding buffer. Then, 100 μL of cell suspension was collected and mixed with 5 μL of Annexin V-FITC and 5 μL of PI, followed by incubation at room temperature for 15 min in dark. After addition of 400 μL of 1× binding buffer, cells were subjected to flow cytometry for the detection of apoptosis rate.

Evaluation of autophagy by transmission electron microscopy

Cells were seeded into 96-well plates and treated with drugs as abovementioned (2.3). Cells were harvested by centrifugation at 1000 r/min for 5 min and then fixed in 2.5% glutaraldehyde and 1% osmium tetroxide. After dehydration in ethanol, permeability and embedding, ultrathin sections were obtained and then stained with lead citrate solution for 15 min and with uranyl acetate in 50% ethanol for 15 min. The ultrastructure of cells was observed under a transmission electron microscope.

Detection of expression of autophagy and apoptosis related proteins by Western blot assay

Cells were seeded into 96-well plates and treated with drugs as abovementioned. Total protein was extracted from cells in each group and protein concentration was determined with BCA method. Then, 50 μg of proteins were subjected to 12% PAGE-SDS and then transferred onto PVDF at constant 250 mA. The membrane was blocked in 3% BSA for 1 h and then incubated with anti-LC3 (1:1000), anti-p62/SQSTM1 (1:1000), anti-cleaved caspase3 (1:1000)
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Influence of SKI on the proliferation of TT cells

As shown in Table 1, SKI treatment for 24 h significantly inhibited the proliferation of TT cells as compared to control group (P<0.05), and this inhibition was dependent on the SKI dose.

**Results**

Influence of SKI on the expression of apoptosis and autophagy related proteins in TT cells

The apoptosis rate (early apoptosis and late apoptosis) increased significantly after 24-h treatment with SKI at 2 and 4 μg/mL (24.3%±5.1% and 47.4%±6.3%, respectively) as compared to blank control group (6.7%±4.2%; P<0.05), and the increase in apoptosis rate was dependent on the dose of SKI. The apoptosis rate of TT cells is shown in Figure 1.

Western blot found that after 24-h treatment with SKI, the expression of cleaved caspase3 (activated caspase 3) and cleaved PARP (activated PARP) increased significantly, and this increase was dependent on the dose of SKI (Figure 2).

**Statistical analysis**

Statistical analysis was performed with SPSS version 20.0. Data are expressed as mean ± standard deviation (x±s). Comparisons were done with ANOVA between groups. A value of P<0.05 was considered statistically significant.

Influence of SKI on the expression of autophagy and apoptosis related proteins

Morphologically, the autophagic cells showed typical bilayer autophagosomes. After 24-h treatment with 4 μg/mL SKI, TEM showed the number of bilayer autophagosomes increased significantly, and only a few autophagic vesicles were observed in control group (Figure 3).

The C terminal of LC3 is cleaved by ATG4 to form LC3-1 which then is coupled to phosphatidylethanolamine (PE) via ATG7 and ATG3 to

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**Figure 1.** SKI induces apoptosis of TT cells (Flow cytometry). *P<0.05 vs control group.

**Figure 2.** Expression of cleaved caspase3 and cleaved PARP in TT cells treated with SKI.
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Autophagy inhibition increases the SKI induced suppression of TT cell proliferation

Form LC3-II. LC3-II localizes in the outer membrane of the autophagosomes, is involved in the prolongation and closure of autophagy precursor membrane and has been an important molecular marker of autophagy [8]. p62/SQSTM1 is a protein that can interact with LC3. The p62/SQSTM1 expression is negatively related to the activity of autophagy [9]. Western blot assay showed the transformation from LC3-I to LC3-II increased with the increase in SKI concentration, but the p62/SQSTM1 expression reduced with the increase in SKI concentration (Figure 4).

Expression of autophagy related proteins increases after exposure to SKI.

Discussion

Shikonin is a natural naphthaquinone compound from the herb Lithospermum erythrorhizon and has been used as a food additive in many countries and has favorable toxicity, pharmacokinetic and pharmacodynamic profiles [10]. It has anti-inflammatory, anti-oxidative, neuroprotective, cardioprotective, anti-tumor and anti-microbial activities [3, 10]. It is known to act on a variety of molecular targets associated with carcinogenesis and shows similar potency towards drug sensitive and drug-resistant cancer cell lines [11]. Chemical agents with potent differentiation-inducing or apoptosis-inducing activity, but acceptable toxic side effects have the potential to be used as anti-cancer drugs. A number of studies suggest that shikonin derivatives may meet this criterion [12]. SKI can induce the apoptosis of cancer cells, inhibit DNA topoisomerases in cancer cells, has anti-mitogenic activity and chemopreventive properties and is able to exert anti-angiogenic effect in cancers [12].

Studies have confirmed that SKI is able to inhibit the cell growth of cancers including leukemia, breast cancer, liver cancer and skin cancer, may regulated some signaling pathways (such as ERK/JNK/MAPK, PI3K/AKT, JAK/STAT3 and those related to endoplasmic reticulum stress [13]) associated with cell survival and proliferation or other biochemical processes to kill cancer cells [4-9, 14]. Our results showed the growth of more than 50% of TT cells was inhibited and the apoptosis rate was 47.4%±6.3% when the SKI concentration was 4 μg/mL. In the apoptosis related signal transduction, caspase-3 is a downstream actor and exerts as an executive signal in the process of
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Apoptosis. When the caspase3 in the plasminogen form is cleaved into active dimer cleaved caspase3 (17 kD/19 kD), the substrates (such as PARP and DNA-PK) are inactivated by being cleaved, leading to cell apoptosis [15, 16]. PARP is hydrolyzed by cleaved caspase3 into 89 kD and 24 kD fragments which are unable to repair the injured DNA, and then the activity of endonuclease negatively regulated by PARP increases, leading to the degradation of nucleosome DNA and subsequent apoptosis [17]. There is evidence showing that SKI is also able to induce necroptosis in cancer cells [18]. Our results indicated that the expression of cleaved caspase3 and cleaved PARP increased significantly after SKI treatment, indicating that SKI induced apoptosis of TT cells is associated with caspase-dependent apoptosis pathway. In the study of Shahsavari et al, shikonin induced a typical caspase-3 dependent apoptosis in T-47D cell line [18]. In cervical cancer cells, a derivative of shikonin was found to induce their apoptosis via PI3K/AKT/mTOR signaling pathway [19]. Moreover, it was found to enhance the cisplatin-induced apoptosis of colon cancer cells, suggesting that shikonin may increase the sensitivity of cancer cells to chemotherapeutics [20]. Besides the apoptosis inducing activity, SKI is also able to inhibit the LPS-induced epithelial-to-mesenchymal transition in human breast cancer cells [21]. In breast cancer, there is evidence showing that SKI can reduce the cancer stem cell load, tumorigenic potential and metastasis [22].

Studies have shown that SKI is able to induce the autophagy of liver cancer cells and pancreatic cancer cells [6, 23]. Autophagy is a highly dynamic processes and has dual roles in the occurrence and development of cancers [24, 25]. 1) over-active autophagy may cause autophagic cell death; 2) the autophagy may degrade proteins with long half-life or cytoplasmic organelles to provide energy for cells and facilitate the turnover of organelles, leading to cell growth. In the present study, our results showed SKI was able to induce the autophagy of MTC TT cells, but whether autophagy increases the anti-tumor activity of SKI or induces the resistance of cancer cells. CQ is a specific autophagy inhibitor and may inhibit the fusion between autophagosomes and lysosomes, leading to the increase in intracellular autophagosomes [26]. Our results indicated
that SKI in combination with CQ significantly increased the apoptosis rate as compared to SKI alone and CQ alone (the apoptosis rate increased from 48.2%±5.8% in SKI group to 62.5%±5.2%). In addition, the protein expression of cleaved caspase3 and cleaved PARP in SKI+CQ group was also markedly higher than in SKI group and CQ group. This suggests that autophagy may reduce the sensitivity of TT cells to SKI and inhibition of autophagy may increase the SKI induced killing of TT cells. This also confirmed the hypothesis that autophagy is involved in the drug resistance of cancer cells and autophagy inhibition may elevate the sensitivity of cancer cells to chemotherapeutics.

Taken together, SKI is able to inhibit the proliferation of MTC TT cells, SKI induced autophagy of TT cells increases their resistance to SKI, and autophagy inhibition can facilitate the SKI induced apoptosis of TT cells. However, the molecular mechanisms underlying the SKI induced apoptosis and autophagy of TT cells and whether to interfere with autophagy at different levels is able to further increase the antitumor activity of SKI are required to be confirmed in more studies.

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Disclosure of conflict of interest

None.

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References

[1] Raue F and Frank-Raue K. Long-Term Follow-up in Medullary Thyroid Carcinoma. Recent Results Cancer Res 2015; 204: 207-225.
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