Effect of scopoletin on PC₃ cell proliferation and apoptosis

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KEY WORDS scopoletin; Lycium barbarum; prostate hyperplasia; cultured cells; cell division; apoptosis; flow cytometry; fluorescence microscopy

ABSTRACT

AIM: To investigate the effect of scopoletin on cell proliferation and apoptosis of PC₃ cells. METHODS: Cell growth curve, MTT assay, and acid phosphatase activity (ACP) were used to determine cell proliferation. Coomassie brilliant blue assay was used to measure the content of protein in cells. Light microscope, transmission electronmicroscope, and fluorescence microscope were used to observe scopoletin-induced morphological changes. Apoptosis rate and cell cycle distribution were determined by flow cytometry. RESULTS: The IC₅₀ of scopoletin for inhibiting PC₃, PAA, and Hela cell proliferation was (157 ± 25), (154 ± 51), and (294 ± 100) mg/L, respectively. Scopoletin induced a marked time- and concentration-dependent inhibition of PC₃ cell proliferation. Scopoletin reduced the protein content and decreased the ACP level in PC₃ cells in a concentration-dependent manner. Cells treated by scopoletin showed typical morphologic changes of apoptosis by light microscope, fluorescence microscope, and transmission electronmicroscope. Apoptosis rate was 0.3 %, 2.1 %, 9.3 % and 35 % for scopoletin 0, 100, 200, and 400 mg/L, respectively, and cells in G2 phase decreased markedly after being treated with scopoletin. CONCLUSION: Scopoletin inhibited PC₃ proliferation by inducing apoptosis of PC₃ cells.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a disease of

old men, and over 50 % of men older than 50 years have been found, at autopsy, to have histologic evidence of prostatic enlargement. With advancing age, there is a progressive increase in the incidence of the disease. Until recently, transurethral resection of the prostate represented the only recognized treatment for BPH. The medical therapies including female hormone, α-receptor blockade, and 5α-reductase inhibitor, etc., have different side effects¹. Therefore to search a more effective medicine without any side effect is of most importance. Recently with the advanced study of apoptosis, it is reported that the apoptotic index of the prostatic epithelium cells is lower in BPH tissue than that in the normal prostate, whereas there is a marked increase in the proliferative index in the hyperplastic prostate. Therefore to increase apoptosis and decrease proliferation is the aim of treatment of BPH²,³.

BPH and primary prostate cancer are common diseases in old men and both diseases are sometimes presented in same gland. It seems that abnormal androgen is especially related with both of them⁴. Since human prostate cancer cell line grows more quickly and it consistently reacts to treatment, it is often used to study the effect of anti-BPH drug⁵. We have found scopoletin is the active component from the fruit of Lycium barbarum for inhibiting prostatic cell proliferation⁶. In this paper, We further investigated the effects of scopoletin on PC₃ cells (human androgen-independent prostate adenocarcinoma cell) both in proliferation and apoptosis.

MATERIALS AND METHODS

Materials Scopoletin, Trypan blue, MTT, RNase, and propidium iodide (PI) were purchased from Sigma Chemical Co. Dulbecco’s modified Eagle’s medium (DMEM) and trypsin were purchased from Gibco. Acridine orange (AO) was obtained from Edward Gurr. Coomassie brilliant blue G-250 was purchased from Serva. New-born calf serum was purchased from Hangzhou Siqing Co. All other
reagents were of analytical reagent quality.

Drug Scopoletin was dissolved in dimethyl sulfoxide (Me₂SO) to make a stock solution, which was then diluted as desired with the culture media (without new-born calf serum). The Me₂SO concentration was kept under 0.001 % in all the experiments and did not show any detectable effect on cell growth or apoptosis.

Cell culture and IC₅₀ PC₃ cells were obtained from ATCC (American Type Culture Collection). PAA (human lung cancer cell) and Hela cells (human cervical squamous cell carcinoma) were kind gifts of Oncology Research Institute of Zhejiang University. All cells of above cell lines were cultured at 37 °C in a humidified CO₂ (5 %) incubator in DMEM supplemented with 10 % heat-inactivated new-born calf serum and passaged at intervals of 3 – 4 d. For all experiments, cells were treated with various concentrations of scopoletin for 24 h after being seeded. The 50 % inhibitory concentrations (IC₅₀) of scopoletin on PC₃, PAA, and Hela cells were determined by MTT assay. The experiment was performed at least three times.

Cell growth curve of scopoletin PC₃ cells (5 × 10³/L) 1 mL in exponential growth were seeded into four 24-well plates (NUNC). The plates were incubated at 37 °C in a humidified 5 % CO₂ atmosphere. After 24 h, scopoletin 33, 66, 133, 266, and 533 mg/L were added to wells (3 wells for each concentration for each plate). For control cells (3 wells for each plate), only DMEM was added. The plates were incubated continually. The viable cells were counted by hemocytometer every day in the frist 4 d by Trypan blue dye exclusion method.

Cell protein determination The cell protein of PC₃ cells treated with or without scopoletin was measured as described previously.

Assay for acid phosphatase (ACP) The activity of acid phosphatase was determined by modified phenyl phosphate method of Kind and King and modified method of Lowry. Briefly, PC₃ cells were treated with scopoletin 0, 100, and 200 mg/L at 24 h after plating. After 72 h, 1 × 10⁶ cells for each concentration were harvested and washed by PBS, then the cell pellets reacted with 0.05 % Triton X-100 0.5 mL on ice bath for 30 min, which resulted in nude enzyme solution. The above solution 60 μL reacted with citrate buffer 38 mmol/L (pH 4.8, containing 0.1 % Triton X-100 and p-nitrophenylphosphata 4.2 mmol/L) at 37 °C exactly for 15 min. Then NaOH 0.1 mol/L 1.2 mL was added to stop the reaction. After being kept at 23 °C – 25 °C for 30 min – 60 min, absorbance at 405 nm was measured with an UV-754 spectrophotometer. Protein content was measured with the Folin phenol reagent by Lowry's method. According to the amount of released p-nitrophenol and the protein content, the ACP activity was calculated (mmol·min⁻¹·g⁻¹ protein).

Morphological determination of apoptosis Light microscopic and fluorescence microscope observation After a 4-d exposure to scopoletin 0, 100, 200, 400 mg/L, the cover slides in each culture dish were taken out, washed with PBS, stained with HE or 0.4 % AO, and put upside down onto a slide prior to microscopic evaluation. The cells were observed under the light microscope and the fluorescence microscope.

Electron microscopic observation After a 4-d exposure to scopoletin 0, 100, 200 mg/L, cells (1 × 10⁶) were fixed with 2.5 % glutaraldehyde, and postfixed with 2 % osmium tetroxide. After dehydration, the samples were embedded in Epon 812, and then ultramicrotomed. The sections were routinely stained and examined by electron microscope.

Flow cytometric analysis of cell cycle After a 3-d treatment with scopoletin, cellular DNA content was detected by flow cytometry via determination of PI. Briefly, after trypsinization, cells (1.0 × 10⁶ per sample) were washed with PBS and cell pellets were fixed in 70 % ethanol at 4 °C overnight. After being washed twice with PBS, RNase (the final concentration was 50 mg/L) was added. After reacting at 37 °C for 60 min, the cells were stained with PI (the final concentration was 50 mg/L) at 4 °C in dark for 60 min before cytofluorometry.

Statistics Data were expressed as x ± s, and compared with t-test. IC₅₀ was calculated by NDST program. Flow cytometry (FCM) results were expressed as percentages and statistical comparisons were made with t test. P < 0.05 is considered significant.

RESULTS

Effect of scopoletin on cell proliferation According to MTT assay, the IC₅₀ of scopoletin for inhibiting PC₃, PAA, and Hela cell proliferation were (157 ± 25), (154 ± 51), and (294 ± 100) mg/L, respectively. Scopoletin induced a marked concentration-dependent inhibition of PC₃ cell proliferation (Fig 1). Scopoletin reduced the protein content in PC₃ cells...
Fig 1. Effect of scopoletin on PC3 cell growth. n = 3 experiments. x ± s. *P < 0.01 vs control (0 mg/L).

in a concentration-dependent manner (Tab 1) and markedly reduced ACP activity in PC3 cells (Tab 2).

Tab 1. Effects of scopoletin on PC3 cells protein content. n = 3 experiments. x ± s. *P > 0.05. **P < 0.05, ***P < 0.01 vs control.

<table>
<thead>
<tr>
<th>Concentration/ mg·L⁻¹</th>
<th>Absorbance</th>
<th>Reduced protein/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.310 ± 0.003</td>
<td>7.8</td>
</tr>
<tr>
<td>33</td>
<td>0.29 ± 0.03</td>
<td>13.2</td>
</tr>
<tr>
<td>66</td>
<td>0.269 ± 0.022</td>
<td>29.1</td>
</tr>
<tr>
<td>133</td>
<td>0.194 ± 0.017</td>
<td>37.2</td>
</tr>
<tr>
<td>266</td>
<td>0.030 ± 0.016</td>
<td>90.4</td>
</tr>
</tbody>
</table>

Tab 2. Effect of scopoletin on ACP in PC3 cells. n = 2 experiments. x ± s. *P < 0.05, **P < 0.01 vs control.

<table>
<thead>
<tr>
<th>Concentration/ mg·L⁻¹</th>
<th>10⁻³ × mmol·min⁻¹·g⁻¹</th>
<th>Inhibition rate/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.06 ± 0.21</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>12.38 ± 0.17</td>
<td>30.8</td>
</tr>
<tr>
<td>200</td>
<td>7 ± 3</td>
<td>65.4</td>
</tr>
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Effect of scopoletin on apoptosis of PC3 cells

After exposure to scopoletin, under both light microscopic and fluorescence microscope, PC3 cells showed typical apoptosis features: volume reduction, chromatin condensation, nuclear fragmentation, and appearance of apoptotic bodies (Fig 2). Under electron microscopy, the chromatin of PC3 cells was located along the nuclear envelope, or formed irregularly shaped crescents at the nuclear edges (Fig 3).

Fig 2. Morphological change of PC3 cells stained by propidium iodide under fluorescence microscope. A: untreated cells; B: cells treated with scopoletin 100 mg/L. × 270.

FCM was accomplished using a FACScan and repeated for 3 times. Nearly 1 × 10⁶ cells were analyzed by excitation and emission of PI at 488 nm and 575 nm, respectively. Examination of untreated PC3 cells by FCM revealed essentially a population of cells that had a normal rate of apoptosis. After treatment of PC3 cells with scopoletin, a subdiploid peak (apoptotic peak) of DNA characteristic of apoptosis was observed. After
Fig 3. Morphological change of PC3 cells under electron microscopy. A: untreated cells (×2500); B: cells treated with scopoletin 100 mg/L (×6000).

The molecular mechanisms that regulate these two processes may underline the abnormal growth of the gland leading to BPH. The apoptotic index of the secretory and basal cells of the prostate epithelium was lower in BPH tissue than that in the normal prostate, whereas there was a marked increase in the proliferative index in the hyperplastic prostate\(^{[2,3]}\). Therefore BPH could be treated by increasing apoptosis and decreasing proliferation. Kyprianou et al found that mean apoptotic indices greatly increased after 3-month treatment of doxazosin, an a blocker, in the glandular epithelial and smooth muscle cells. By 12 months after treatment epithelial apoptosis had decreased to constitutive levels, while the apoptotic index of prostatic stroma cells remained high. Doxazosin induced apoptosis of smooth muscle cells which correlated with prostatic stromal degeneration, and decreased e-smooth muscle actin expression and improved BPH symptoms\(^{[10]}\). Sez et al reported that finasteride, a 5a-reductase inhibitor, might modulate the TGF-β (transforming growth factor β) signaling system to promote changes leading to apoptosis of epithelial cells and prostatic glandular atrophy\(^{[11]}\). In our experiment, based on FCM, the apoptosis index of PC3 cells was increased with scopoletin concentration. Previous reports indicated that when ACP activity decreased, the protein synthesis was inhibited in prostate\(^{[8,9]}\). Our results indicated ACP activity and protein content in PC3 cells were decreased with scopoletin concentration. According to PC3 cell growth curve, the protein content and ACP activity in PC3 cells, it was found that scopoletin inhibited PC3 cell proliferation. The PC3 cells were mainly arrested in G0/G1 and S phases. Therefore scopoletin might be possessed of anti-benign prostatic hyperplasia actions both by decreasing proliferation and increasing apoptosis of prostatic cells.

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REFERENCES


1 蒿芩对 PC3 细胞增殖和凋亡的影响

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关键词: 蒿芩; 枸杞子; 前列腺增生; 培养的细胞; 细胞分裂; 细胞凋亡; 流式细胞术; 反光显微镜检查

目的: 研究蒿芩对人前列腺癌细胞 PC3 增殖的作用和蒿芩是否能引起 PC3 细胞的凋亡。方法: 用细胞生长曲线、MTT 试验和酸性磷酸酶 (ACP) 活性来测定细胞增殖, 检测细胞内蛋白质的含量, 光镜, 透射电镜和荧光显微镜观察蒿芩对 PC3 细胞的形态学变化。用流式细胞仪确定细胞周期和细胞的周期分布。结果: 蒿芩对 PC3 细胞的 

PAA 和 Hela 细胞的 IC50 分别为 (157 ± 25), (154 ± 51) 和 (294 ± 100) mg/L, 蒿芩对 PC3 细胞时间和浓度依赖性地抑制 PC3 细胞的增殖, 并引起细胞内蛋白质含量减少和 ACP 活性降低。经蒿芩处理后, 在光镜、透射电镜和荧光显微镜下可观察到蒿芩对 PC3 细胞的典型凋亡形态学变化, 流式细胞仪检测显示经蒿芩 0, 100, 200 和 400 mg/L 处理后, PC3 细胞的凋亡率分别为 0.3 %, 2.1 %, 9.3 % 和 35 %, 各细胞显著减少。结论: 蒿芩抑制 PC3 细胞增殖且可引起 PC3 细胞的凋亡。