Influence of α-Asarone on Human Esophageal Carcinoma Cells and Its Molecular Mechanisms

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Citation

Received: May 9, 2018; Accepted: June 12, 2018; Published: June 28 2018

Abstract: α-Asarone - the main component of the rhizomes of Acori graminei that has been traditionally used in the treatment of nervous system disorders. The present work envisages an investigation of the influence of α-asarone on human esophageal carcinoma cells (Eca-109), explore its molecular mechanism, and provide an experimental basis for the clinical application. The methyl thiazolyl tetrazolium (MTT) assay was performed to detect the cell proliferation after cells were processed with α-asarone for various time points. The morphological changes of the apoptotic cells stained with AO/EB were observed with an inverted microscope. After 48 hours of in vitro culturing, the expression levels of GRP78, CHOP, caspase-3 and caspase-9 genes and proteins were measured by RT-PCR and Western blot technique. MTT data showed that α-asarone inhibited the cell proliferation in a time and dose-dependent manner. In addition, α-asarone induced apoptosis in the Eca-109 cells and increased the expressions of GRP78, CHOP, caspase-3 and caspase-9 significantly. The present study demonstrated that the apoptosis was mediated by an up-regulation of expressions of the endoplasmic reticulum-associated GRP78, CHOP, and cysteine proteinase caspase-3, caspase-9. This provides an experimental basis for clinical application of α-asarone in anti-tumor therapy, however further investigations of the in vivo experimentation need to be done.

Keywords: α-Asarone, Apoptosis, GRP78, CHOP, Caspase-3, Caspase-9

1. Introduction

Esophageal carcinoma, which is characterized by a high morbidity, is the second leading cause of deaths due to a malignant tumor of the digestive tract in China [1]. Generally, the primary tumor of most patients is cured with surgical resection, systemic chemotherapy, and radiation therapy; however, many patients eventually succumb to the disease [2]. Therefore, determining a new alternative treatment for improving the survivability is very essential. There are many Traditional Chinese medicines and some Chinese herb extracts for prevention or treatment of cancer by inducing tumor cell apoptosis. There is a need to find plant extracts with low toxicity and high efficacy in the treatment of cancer [3].

α-Asarone is the main component of the rhizomes of Acori graminei which is frequently used for central nervous system disorders and cognitive dysfunction [4]. Previous studies have proved α-asarone to possess extensive pharmacological actions including anti-gastric ulcer and anti-allergic activities, inhibition of histamine release and antioxidiant effect, and promotion of angiogenesis [5]. However, its effect on human esophageal carcinoma cell line Eca-109 remains largely unknown. Therefore, the present study aimed to investigate the proliferation and apoptosis effect of α-asarone on esophageal carcinoma Eca-109 cells. In our former work, we had discovered the correlation between α-asarone and tumor cells. α-Asarone could inhibit Eca-109 cell proliferation along with the induction of apoptosis [6]. Therefore, in this study, we tried to clarify the mechanism of α-asarone on Eca-109 cells.

2. Methods

2.1. Drugs and Reagents

α-Asarone was purchased from Shanxi Powerdone
Pharmaceutics Co., Ltd.(201308), DaTong, China. Fetal bovine serum was obtained from Hangzhou Sijiqing Biotechnology Co., China. AO/EB fluorescent dyes kits were purchased from Beijing Solarbio Science & Technology Co., Ltd., China; Mouse anti-human monoclonal antibodies to β-actin (Santa cruz, SC81178), Mouse anti-human polyclonal antibodies to GRP78 (Abcam ab151269), CHOP (Cell Signaling L63F7), Caspase-3 (Abcam ab119794), Caspase-9 (Abcam ab115792), RNA extraction kit and reverse transcription kit (TaKaRa), UltraSYBR Mixture (Beijing Kangwei Biological Technology Co., Ltd.).

2.2. Cells Culture and Experimental Groups

Eca-109 cells line was procured from the Basic Research Laboratory of the Basic Medical College, Henan University of Traditional Chinese Medicine (HUTCM). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin, in an incubator with 5% CO₂. 2.5. Expression of GRP78, CHOP, Caspase-3 and Caspase-9 genes. 

2.4. Cell Apoptosis Using Immunofluorescence Staining

Experiments were performed according to the AO/EB kit instructions. Briefly, cells were treated with different concentrations of α-asarone for 48h and were washed twice by PBS. AO solution and EB solution were mixed into working liquid in 1:1 ratio. Then cells were dyed with the working liquid and the morphological changes of the cells treated with α-asarone were observed under inverted fluorescence microscope (IX73, Olympus) [7].

2.5. Expression of GRP78, CHOP, Caspase-3 and Caspase-9 mRNA by Real-Time QPCR

After treatment with α-asarone for 48h, the total RNA of cells was extracted using TRIzol reagent. The cDNA was converted using the reverse transcription kit. The primers for human GRP78, CHOP, caspase-3, caspase-9, and β-actin were designed using Primer Express displays as follows. β-actin: F: 5'-CCGTCTCTCCCTCTCCATCG-3', R: 5'-GTCCACGTATGGTAGAATGC-3', product length 155bp; GRP78: F: 5'-GAACACAGTTGCGCTACCAAGA-3', R: 5'-TCCAGTCAGTCAAATGTACCCAGA-3', product length 142bp; CHOP: F: 5'-GGGAAAACCGCATGAAGGA-3', R: 5'-GGCTGATGTTGCTGGTAC-3', product length 221bp; Caspase-3: F: 5'-CAGTGGAGGCCGACTTCTTG-3', R: 5'-TGTTGACAAACGGACTGGAT-3', product length 101bp; Caspase-9: F: 5'-CTGCGAAGTACACAGGAAGGC-3', R: 5'-CTAGATATGGCGTCCAGCT-3', product length 286bp. Human β-actin expression was used as internal control. The 2ΔΔCt method was adopted to calculate the relative expression of the GRP78, CHOP, Caspase-3 and Caspase-9 genes. ΔΔCt = ΔCt (target sample) - ΔCt (control sample), ΔCt = CT (test gene) – CT (reference gene) [13]. Each sample was tested in triplicate. PCR reaction total volume was 10µL that contained: SYBR Green 1 Mixture 5µL, upstream primer 0.25µL, downstream primer 0.25µL, Template cDNA1µL, ddH₂O 3.3µL, and Rox0.2µL. Reaction conditions were: 40 cycles, initial denaturation at 95°C for 10min, denaturation at 95°C for 30s, annealing at 58°C for 40s, extension at 72°C for 40s.

2.6. Expression of the GRP78, CHOP, Caspase-3 and Caspase-9 Protein by Western Blot

Eca-109 cells treated with α-asarone for 48h were rinsed twice with ice-cold PBS and lysed in Cell Lysis Buffer for 50min on ice, centrifuged at 12000g for 20min at 4°C, and the supernatant fluid was collected. Protein concentration was measured by the BCA method. 30µg of protein was loaded for Western blot analysis. Briefly, proteins were electrophoresed on SDS-polyacrylamide gel electrophoresis and electro-transferred to a PVDF membrane, which was blocked with 5% non-fat milk for 2h at ambient temperature. Membranes were then incubated with mouse anti-human GRP78, CHOP, Caspase-3 and Caspase-9 antibodies (1:1000) overnight at 4°C. Then the secondary antibody IgG (1:1000) was applied. All protein bands were detected by chemiluminescence detection system.

2.7. Statistical Analysis

Each experiment was replicated for at least three times. All statistical analyses were conducted by the software SPSS 17.0. Results are shown as the mean ±SD, and the comparisons between the control and the experiment were made using one-way ANOVA test. The statistical significance was defined as a value of P < 0.05.

3. Results

3.1. Effects of α-Asarone on Cells Proliferation

The cells proliferation inhibition induced by α-asarone was...
assessed by MTT after 12, 24, 36 and 48h of drug exposure. As shown in Figure 1, with the extension of time (12 h ~ 48 h) and the augment of dose (0.025 mg/mL ~ 0.1 mg/mL), the inhibition rate gradually increased from 0% to 71%. The highest inhibition rate reflected in a concentration of 0.1mg/mL at 48h. Thus, the growth of cells was obviously inhibited along with a dose- and time-dependent manner (P < 0.05).

Figure 1. Inhibitory effects of different concentration α-asarone (0.025, 0.05, 0.1 mg/mL) on Eca-109 cells at different time points. Results showed that Eca-109 cells proliferation demonstrated a dose- and time- dependence on α-asarone exposure at the 0.025 mg/mL ~ 0.1 mg/mL from 12 h to 48 h. Compared with the control group, the discrepancy of cell proliferation inhibition via α-asarone shows remarkable statistical significance,*p < 0.05.

3.2. Effects of α-Asarone on Eca-109 Cells Morphology and Apoptosis

A significant apoptosis was observed after the treatment of the Eca-109 cells with α-asarone for 48h, with substantial morphological changes. After AO/EB double fluorescence staining, fluorescence microscopy revealed that the morphology of cell nuclear in the control group was normal and nuclear staining presented uniformly green fluorescence, while in the experimental group, most of the cell nuclei underwent pyknosis and presented orange fluorescence (early apoptosis state) or red fluorescence (middle-late apoptosis state) (Figure 2).

Figure 2. The influence of different concentration α-asarone (0.025, 0.05, 0.1 mg/mL) on Eca-109 cells morphology and apoptosis under inverted fluorescence microscope (Magnification ×200). After all cells were dyed with AO/EB double fluorescence staining, the results were as follows: Green fluorescence signified living cells; Orange fluorescence signified early apoptosis cells; Red fluorescence signified middle-late apoptotic cells. We found that the cells treated by 0.05 and 0.1 mg/mL concentration α-asarone displayed obvious orange and red fluorescence.

3.3. Effects of α-Asarone on Expression Levels of GRP78, CHOP, Caspase-3 and Caspase-9 mRNA

In this study, the expression of GRP78 and CHOP mRNA significantly were increased when Eca-109 cells were exposed to 0.025 mg/mL ~ 0.1 mg/mL (P < 0.05) (Table 1, Table 2). At the same time, the cells that were exposed to 0.05 mg/mL ~ 0.1mg/mL α-asarone also up-regulated the expression of caspase-3 and caspase-9 mRNA whose expressions level were significantly higher than in the control group (P < 0.05) (Table 3, Table 4). However, the expression levels of caspase-3 and caspase-9 mRNA exhibited no obvious difference when cells were treated with α-asarone with 0.025 mg/mL concentration.

3.4. Effects of α-Asarone on Protein Expression Levels of GRP78, CHOP, Caspase-3 and Caspase-9

The protein expression of GRP78, CHOP, caspase-3 (at 0.05mg/mL and 0.1mg/mL concentration) and.

Table 1. The CT value of α-asarone on the gene expression of GRP78 in Eca-109 cells.

<table>
<thead>
<tr>
<th>group</th>
<th>CT value (GRP78)</th>
<th>CT value (β-actin)</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>2^ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>20.37±0.17</td>
<td>16.97±0.12</td>
<td>3.4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>α-asarone 0.025 mg/mL</td>
<td>19.13±0.01</td>
<td>17.30±0.16</td>
<td>1.83</td>
<td>-1.57</td>
<td>2.97*</td>
</tr>
<tr>
<td>α-asarone 0.05 mg/mL</td>
<td>18±0.34</td>
<td>17.24±0.21</td>
<td>0.75</td>
<td>-2.65</td>
<td>6.28*</td>
</tr>
<tr>
<td>α-asarone 0.1 mg/mL</td>
<td>17.67±0.15</td>
<td>17.13±0.01</td>
<td>0.54</td>
<td>-2.86</td>
<td>7.267*</td>
</tr>
</tbody>
</table>

Note: ΔCt = Ct (target gene) - Ct (reference gene), ΔΔCt = ΔCt (experimental group) - ΔCt (control group). Compared with the control group, 2^ΔΔCt signify that the expression of target gene of experimental group occurred change.

Table 2. The CT value of α-asarone on the gene expression of chop in Eca-109 cells.

<table>
<thead>
<tr>
<th>group</th>
<th>CT value (chop)</th>
<th>CT value (β-actin)</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>2^ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>25.72±0.18</td>
<td>16.97±0.12</td>
<td>8.75</td>
<td>0</td>
<td>1</td>
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<tr>
<td>α-asarone 0.025 mg/mL</td>
<td>24.06±0.42</td>
<td>17.30±0.16</td>
<td>6.76</td>
<td>-1.99</td>
<td>3.9782*</td>
</tr>
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<td>α-asarone 0.05 mg/mL</td>
<td>22.94±0.29</td>
<td>17.24±0.21</td>
<td>5.7</td>
<td>-3.05</td>
<td>8.28*</td>
</tr>
<tr>
<td>α-asarone 0.1 mg/mL</td>
<td>22.8±0.05</td>
<td>17.13±0.01</td>
<td>5.67</td>
<td>-3.08</td>
<td>8.46*</td>
</tr>
</tbody>
</table>

Note: idem
### Table 3. The CT value of α-asarone on the gene expression of caspase-3 in Eca-109 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>CT value (caspase-3)</th>
<th>CT value (β-actin)</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>2^ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.35±0.05</td>
<td>16.97±0.12</td>
<td>9.38</td>
<td>0</td>
<td>1</td>
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<tr>
<td>α-asarone 0.025 mg/mL</td>
<td>26.6±0.46</td>
<td>17.3±0.16</td>
<td>9.3</td>
<td>-0.08</td>
<td>1.06</td>
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<td>α-asarone 0.05 mg/mL</td>
<td>26.21±0.15</td>
<td>17.24±0.21</td>
<td>8.97</td>
<td>-0.41</td>
<td>1.33*</td>
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<tr>
<td>α-asarone 0.1 mg/mL</td>
<td>26.28±0.06</td>
<td>17.13±0.01</td>
<td>9.15</td>
<td>-0.23</td>
<td>1.173*</td>
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</table>

Note: idem.

### Table 4. The CT value of α-asarone on the gene expression of caspase-9 in Eca-109 cells.

<table>
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<tr>
<th>Group</th>
<th>CT value (caspase-9)</th>
<th>CT value (β-actin)</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>2^ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.3±0.24</td>
<td>16.97±0.12</td>
<td>9.38</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>α-asarone 0.025 mg/mL</td>
<td>25.89±0.81</td>
<td>17.3±0.16</td>
<td>9.37</td>
<td>-0.01</td>
<td>1.01</td>
</tr>
<tr>
<td>α-asarone 0.05 mg/mL</td>
<td>26.24±0.19</td>
<td>17.24±0.21</td>
<td>8.6</td>
<td>-0.78</td>
<td>1.72*</td>
</tr>
<tr>
<td>α-asarone 0.1 mg/mL</td>
<td>25.96±0.14</td>
<td>17.13±0.01</td>
<td>8.83</td>
<td>-0.55</td>
<td>1.46*</td>
</tr>
</tbody>
</table>

Note: idem.

Table 1. The effect of different concentration α-asarone on the gene expressions of GRP78, CHOP, Caspase-3 and Caspase-9 in Eca-109 cells.

Caspase-9 were increased significantly \((P<0.05)\). The expression of caspase-3 at 0.025mg/mL concentration exhibited no significant difference (Figure 3).

4. Discussion

Cell apoptosis, also known as programmed cell death (PCD), is a physiological process that is precisely initiated and regulated by multiple intrinsic and extrinsic aspects, such as gene activation, expression and signal transduction [1]. At
present, there are three main apoptosis pathways including the death receptor pathway, the mitochondrial pathway, and the endoplasmic reticulum stress pathway. In this study, the mechanism of apoptosis induced by α-asarone on Eca-109 cells was mediated through the endoplasmic reticulum stress pathway in which, the related genes and proteins such as GRP78, CHOP and Caspases are of vital importance.

Alpha (α)-asarone[1,2,4-trimethoxy-5-[(E)-pro-1-enyl]] is one of the main pharmacologically active compounds present in Acorus calamus Linn (Acoraceae), Acorus tatarinowii Schott (Acoraceae), and Acorus gramineus Solander [8]. Traditional Chinese Medicine prefers Acorus gramineus in Chinese herbal medicine for the therapy of epilepsy, which is attributed to the role of dissipating phlegm, inducing resuscitation, reinforcing intelligence, sedation and relieving convulsion [9]. The purpose of this study was to investigate the effect of α-asarone on the apoptosis of Eca-109 cells. We found in our previous preliminary study that α-asarone could inhibit cell proliferation and induce cell apoptosis [10]. In the present study, the endoplasmic reticulum stress pathway plays an important role in the regulation of cell apoptosis. It is well known that the endoplasmic reticulum (ER) is a central organelle involved in the intracellular calcium regulation and protein folding [11]. Low oxygen and low glucose stimulation can make misfolded or unfolded proteins to accumulate in the ER, and lead to its stress, thus inducing apoptosis [12]. Endoplasmic reticulum stress (ERS) regulate cell apoptosis through multiple signaling pathways such as, caspase-12, c-Jun N terminal kinase, Ca²⁺, CHOP [13]. CHOP is the specific transcription factor that induces apoptosis by ERS. It inhibits the Bcl-2 expression and has low expression in normal cells, but in the ERS it gets activated and exhibits a higher protein expression level. The process gets completed by the activation of caspase-9 protease precursor, launching protease cascade reaction, eventually inducing apoptosis [14, 15]. GRP78 participates in the protein folding and transshipment and is secreted by the endoplasmic reticulum. GRP78 plays a very important role in the maturation of cell proteins and maintaining the cell function at normal growth conditions. GRP78 is a kind of endoplasmic reticulum stress protein that gets expressed to a higher level when the cells are stimulated due to the instability of the endoplasmic reticulum [16].

We found that the GRP78 expression decreased when the Eca-109 cells were treated with α-asarone (0.025, 0.05 and 0.1 mg/mL) for 48 hours, which suggests that a decrease in GRP78 expression may lead to the inhibition of tumor cell proliferation, and the induction of tumor cell apoptosis. Thus, α-asarone can induce cell apoptosis by decreasing GRP78 expression and subsequently activating CHOP. The ERS pathways may play an important role in α-asarone induced Eca-109 cell apoptosis.

Current research shows that the expression level of GRP78 in normal cells is higher than that in tumor cells [17]. At the same time, studies have shown that GRP78 may participate in the process of tumor invasion and metastasis [18]. Caspase family is the most important protease of cell apoptosis, of which caspase-3 is a common enzyme in three classic apoptosis pathway [19]. Caspases get specificity activated in ERS and start the cascade process by activating caspase-9 that further activates caspase-3, eventually leading to apoptosis [20].

Our results reveal the pharmacological actions of α-asarone on the esophageal cancer cells Eca-109 by a modulation in the expression of the ERS proteins GRP78 and CHOP, and then specific activation of Caspase-9, which can crack downstream effects enzymes including Caspase-3. Activated Caspase-3 resulted eventually in the cell apoptosis. As expected, there is a close relationship between the proliferation and apoptotic effect of α-asarone on Eca-109 cells and ER marker proteins expression. In other words, α-asarone could induce apoptosis of Eca-109 cells through up-regulation of their protein expressions.

5. Conclusions

In the present study, we initially found that the proliferation of Eca-109 cells treated with α-asarone was significantly inhibited by an induction of apoptosis, which was mediated by the activation of the GRP78-CHOP-caspase pathway. This provides an experimental basis for the clinical application of α-asarone in anti-tumor therapies. However, more understanding about other molecular mechanisms of cells apoptosis due to α-asarone need to be studied. The in vivo experimentation of α-asarone is also required to aid the further research in this arena. Our findings offer evidence that α-asarone could have an immense potential to induce apoptosis, and may be a novel candidate for the treatment of malignant tumors.

Disclosure Statement

The authors declare no conflict of interest.

Funding Sources

This work was supported by College Key Scientific Research Project of Henan province (No. 17A310021).

References


