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Experimental study on the apoptosis of cervical cancer Hela cells induced by juglone through c-Jun N-terminal kinase/c-Jun pathway

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ABSTRACT

Objective: To study the regulatory effect and molecular mechanism of juglone on apoptosis of cervical cancer Hela cells.
Methods: Cervical cancer Hela cells were cultured and treated with different dosages of juglone (10, 20, and 40 µmol/L, respectively) and c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10, 20, and 40 µmol/L, respectively). Then cellular proliferative activity and the expression of JNK/c-Jun pathway molecule and apoptotic molecule in the cells were detected.
Results: After 6, 12, 18 and 24 h of treatment, the value for proliferative activity of cells treated with juglone was significantly lower than that of control group ($P < 0.05$), and the anti-proliferative effect was more significant as the treatment period and juglone dosage increased ($P < 0.05$). The protein expressions of Bax, CytC, Fas, FasL, Caspase-3, p-JNK and p-c-Jun in cells treated with juglone were significantly higher than those of control group ($P < 0.05$), and the protein expressions of Bax, CytC, Fas, FasL, Caspase-3, p-JNK and p-c-Jun increased more remarkably as the juglone dosage increased ($P < 0.05$). In cells treated with 40 µmol/L juglone and SP600125, the protein expressions of Bax, CytC, Fas, FasL and Caspase-3 were significantly lower than those of cells treated with 40 µmol/L juglone ($P < 0.05$), and the protein expressions of Bax, CytC, Fas, FasL and Caspase-3 reduced more remarkably as the SP600125 dosage increased ($P < 0.05$).
Conclusion: Juglone can increase the expression of apoptotic molecules in mitochondrial pathway and death receptor pathway by activating JNK/c-Jun pathway, thus inducing apoptosis of cervical cancer cells.

1. Introduction

Cervical cancer is the most common malignancy of the reproductive system in women, and platinum-based radiotherapy and chemotherapy is a common way to treat advanced cervical cancer. Patients with cervical cancer in the process of radiotherapy and chemotherapy will produce drug resistance, which manifests as reduced sensitivity to radiotherapy and chemotherapy, and tumor progression or recurrence [1,2]. The chemical component of juglone is 5-hydroxy-1,4-naphthalenedione, which is extracted

from the stem, leaf and shell of *Juglans mandshurica* and confirmed to possess anti-tumor activity and exhibit inhibitory effect on the growth of a variety of malignant tumors [3,4]. However, the inhibitory effect and molecular mechanism of juglone on cervical cancer cells growth has not been elucidated yet. c-Jun N-terminal kinase (JNK)/c-Jun pathway is an important pro-apoptotic signaling pathway in the cells. Different extracellular physiochemical factors can activate the JNK/c-Jun signaling pathway and initiate the transcription of a variety of downstream pro-apoptotic molecules, thus inducing apoptosis [5,6]. In the present study, we analyzed whether juglone induces apoptosis of cervical cancer Hela cells by JNK/c-Jun pathway.

2. Materials and methods

2.1. Experimental materials

Cervical cancer Hela cells were purchased from the cell bank of Chinese Academy of Sciences, and DMEM and fetal bovine

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serum used for cell culture and trypsin used for cell passage were purchased from Gibco Company. Juglone was purchased from Guangzhou Aikenman Biotechnology Co., Ltd, JNK inhibitor SP600125 from MEC Company, MTS cell proliferation assay kit from Promega Company, precast gels for Western blot from Absin Company, PVDF membrane from Thermo Company and monoclonal antibody from Abcam Company.

2.2. Grouping and treatment of cells

The cells were cultured in DMEM containing 10% fetal bovine serum and digested with trypsin when cell confluence reached 80%–90%. Then the cells were inoculated in the culture plate and treated with different treatments: (1) control group: treated with DMEM without any agents and serum; (2) juglone groups: treated with serum-free DMEM containing 10, 20 and 40 μmol/L juglone, respectively; (3) SP600125 groups A, B and C: treated with serum-free DMEM containing 40 μmol/L juglone combined with 10, 20 and 40 μmol/L SP600125, respectively.

2.3. Detection of cellular proliferative activity

Hela cells were inoculated in 96-well culture plate and subjected to different treatments for 6, 12, 18 and 24 h. Then the medium was discarded and 20 μL MTS solution was added, followed by incubation for 2–3 h. After incubation, the culture plate was taken and absorbance was read at 450 nm by using a microplate reader. The cellular proliferative activity corresponding to the absorbance value of control group was 100, which was used for calculation of the cellular proliferative activity in other treatment groups.

2.4. Detection of gene expression

The cells were inoculated in 12-well plate, and treated with different treatments for 24 h. Then protein lysis buffer was added and cellular protein was extracted. The loading buffer was added and the protein was subjected to high-temperature denaturation. The denatured protein sample was collected and blotted onto the precast gels, subjected to electrophoresis, and then transferred onto PVDF membrane. Then the PVDF membrane was blocked in 5% skimmed milk for 2–3 h, and the corresponding monoclonal antibody was incubated at 4 °C overnight. The next day, PVDF membrane was taken and HRP-labeled secondary antibody was incubated at room temperature for 2 h. ECL reagent was added and the spots were developed in a chemiluminescence instrument, and the protein band was obtained and gray value scanned. β-actin was used as internal reference to calculate the protein expressions of Bax, CytC, Fas, FasL and Caspase-3; while JNK/c-Jun was used as internal reference to calculate the protein expressions of p-JNK and p-c-Jun.

2.5. Statistical analysis

Data were input and analyzed by using SPSS 20.0 software. Data among groups were compared by using variance analysis, and comparison between any two groups was performed by using *LSD-t* test; difference was statistically significant when $P < 0.05$.

3. Results

3.1. Regulatory effects of juglone on proliferative activity of Hela cells

After 6, 12, 18 and 24 h of the treatment, the cellular proliferative activity of juglone groups (10, 20 and 40 μmol/L) was significantly lower than that of control group ($P < 0.05$), and the anti-proliferative effect was more significant as the treatment period and juglone dosage increased ($P < 0.05$) (Table 1).

3.2. Regulatory effects of juglone treatment on expression of apoptotic molecule in Hela cells

After 24 h of the treatment, the protein expressions of Bax, CytC, Fas, FasL, Caspase-3 in cells of juglone groups (10, 20 and 40 μmol/L) were significantly higher than those of control group ($P < 0.05$), and the protein expressions of Bax, CytC, Fas, FasL, Caspase-3 increased more remarkably as the juglone dosage increased ($P < 0.05$) (Table 2).

3.3. Regulatory effects of juglone treatment on expressions of p-JNK and p-c-Jun in Hela cells

After 24 h of the treatment, the protein expressions of p-JNK and p-c-Jun in cells of juglone groups (10, 20 and 40 μmol/L) were significantly higher than those of control group ($P < 0.05$), and the protein expressions of p-JNK and p-c-Jun increased more remarkably as the juglone dosage increased ($P < 0.05$) (Table 3).

3.4. Effect of juglone combined with JNK inhibitor SP600125 on the expressions of apoptotic molecule in Hela cells

After 24 h of the treatment, the protein expressions of Bax, CytC, Fas, FasL, Caspase-3 in cells of SP600125 groups A, B and C were significantly lower than those of 40 μmol/L juglone group ($P < 0.05$), and the protein expressions of Bax, CytC, Fas,

Table 1
Effect of different dosages of juglone on proliferative activity of Hela cells ($n = 5$, $\bar{x} \pm s$).

Groups	Treatment time (h)			
	6	12	18	24
Control group	1.00 ± 0.15	1.09 ± 0.17	1.24 ± 0.19	1.37 ± 0.22
10 μmol/L juglone group	0.89 ± 0.12[a]	0.80 ± 0.10[a]	0.71 ± 0.09[a]	0.65 ± 0.08[a]
20 μmol/L juglone group	0.74 ± 0.09[ab]	0.68 ± 0.08[ab]	0.57 ± 0.08[ab]	0.41 ± 0.07[ab]
40 μmol/L juglone group	0.61 ± 0.08[abc]	0.47 ± 0.06[abc]	0.35 ± 0.04[abc]	0.29 ± 0.04[abc]

[a]: $P < 0.05$ compared with control group; [b]: $P < 0.05$ compared with 10 μmol/L juglone group; [c]: $P < 0.05$ compared with 20 μmol/L juglone group.

Table 2

Effect of different dosages of juglone on expression of apoptotic molecule in Hela cells ($n = 5$, $\bar{x} \pm s$).

Groups	Bax	CytC	Fas	FasL	Caspase-3
Control group	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.12	1.00 ± 0.18	1.00 ± 0.11
10 μmol/L juglone group	1.45 ± 0.19^{a}	1.64 ± 0.20^{a}	1.39 ± 0.17^{a}	1.52 ± 0.19^{a}	1.59 ± 0.22^{a}
20 μmol/L juglone group	1.98 ± 0.24^{ab}	2.29 ± 0.34^{ab}	1.87 ± 0.22^{ab}	2.14 ± 0.34^{ab}	2.78 ± 0.38^{ab}
40 μmol/L juglone group	2.74 ± 0.35^{abc}	3.41 ± 0.58^{abc}	2.59 ± 0.35^{abc}	3.29 ± 0.51^{abc}	3.89 ± 0.51^{abc}

β-actin was used as internal reference to calculate the protein expressions of Bax, CytC, Fas, FasL and Caspase-3. [a]: $P < 0.05$ compared with control group; [b]: $P < 0.05$ compared with 10 μmol/L juglone group; [c]: $P < 0.05$ compared with 20 μmol/L juglone group.

FasL, Caspase-3 reduced more remarkably as the SP600125 dosage increased ($P < 0.05$) (Table 4).

4. Discussion

Drug resistance is an important factor affecting the chemotherapy outcome and prognosis of patients with cervical cancer. Combination of different anti-tumor drugs with different modes of action is a feasible approach to treat cervical cancer. Juglone is a hydroxynaphthoquinone compound extracted from the stem, leaf and shell of *Juglans mandshurica*, which possesses anti-tumor activity. In recent years, more and more basic researches have confirmed that juglone has an inhibitory effect on the proliferation, migration and invasion of breast cancer, ovarian cancer, pancreatic cancer cells and such malignant tumors [7–9]. It can also induce the apoptosis of stomach cancer and breast cancer cells [10,11]. In order to clarify the effect of juglone on the apoptosis of cervical cancer cells, this study first analyzed the effect of different doses of juglone on the proliferative activity of Hela cells, and the results showed that treatment with juglone significantly reduced the proliferative activity of Hela cells ($P < 0.05$), and the anti-proliferative effect was more significant as the treatment period and juglone dosage increased ($P < 0.05$). This result indicated that juglone

significantly inhibited the proliferation of cervical cancer cells and induced apoptosis of cervical cancer cells.

Mitochondrial apoptotic pathway and death receptor apoptotic pathway are two important pathways of apoptosis [12,13]. Bax is an important molecule for the regulation of mitochondrial apoptotic pathway, which is capable of forming homodimers on the mitochondrial membrane and opening the permeability transition pore on the mitochondrial membrane to promote the release of CytC from mitochondria into the cytoplasm. After entry into the cytoplasm, CytC can activate caspase-9-mediated cascade amplification reaction and ultimately induce apoptosis through the activation of caspase-3 [14,15]. Fas/FasL is an important molecule to regulate the death receptors apoptotic pathway. Fas belongs to the tumor necrosis factor receptor superfamily, which binds to the ligand FasL to form a death-inducing signaling complex, activates caspase-8-mediated cascade amplification reaction, and finally induces apoptosis by activating caspase-3 [16,17]. In this study, the expression of apoptotic molecules in Hela cells treated with juglone was analyzed, and the results revealed that juglone treatment can significantly increase the protein expressions of Bax, CytC, Fas, FasL and Caspase-3 ($P < 0.05$), and the expressions increased more remarkably as the treatment period and juglone dosage increased ($P < 0.05$), thus indicating that juglone activated the mitochondrial apoptotic pathway and death receptor apoptotic pathway of cervical cancer cells.

At present, the molecular pathways of cervical cancer cells apoptosis induced by juglone, and the expression of apoptotic molecules in cervical cancer cells regulated by juglone have not been clarified. JNK/c-Jun pathway is an important pro-apoptotic signaling pathway in the cells. Extracellular physiochemical factors can cause JNK phosphorylation and translocation into the cell nucleus. After entry into cell nucleus, p-JNK can combine with transcription factor c-Jun to induce phosphorylation. The transcriptional activity and stability of p-c-Jun were significantly increased, enabling the initiation of apoptotic molecules transcription in downstream mitochondrial pathways and death receptor pathway [18–20]. Phosphorylation of signaling

Table 3

Effect of different dosages of juglone on expressions of p-JNK and p-c-Jun in Hela cells ($n = 5$, $\bar{x} \pm s$).

Groups	p-JNK/JNK	p-c-Jun/c-Jun
Control group	1.00 ± 0.17	1.00 ± 0.13
10 μmol/L juglone group	1.51 ± 0.19^{a}	1.68 ± 0.20^{a}
20 μmol/L juglone group	1.89 ± 0.23^{ab}	2.39 ± 0.35^{ab}
40 μmol/L juglone group	2.83 ± 0.39^{abc}	3.41 ± 0.49^{abc}

[a]: $P < 0.05$ compared with control group; [b]: $P < 0.05$ compared with 10 μmol/L juglone group; [c]: $P < 0.05$ compared with 20 μmol/L juglone group.

Table 4

Effect of juglone combined with different dosages of SP600125 on the expressions of apoptotic molecule in Hela cells ($n = 5$, $\bar{x} \pm s$).

Groups	Bax	CytC	Fas	FasL	Caspase-3
Control group	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.12	1.00 ± 0.18	1.00 ± 0.11
40 μmol/L juglone group	2.74 ± 0.35^{a}	3.41 ± 0.58^{a}	2.59 ± 0.35^{a}	3.29 ± 0.51^{a}	3.89 ± 0.51^{a}
SP600125 group A	1.98 ± 0.22^{b}	2.29 ± 0.39^{b}	1.89 ± 0.22^{b}	2.33 ± 0.38^{b}	2.41 ± 0.41^{b}
SP600125 group B	1.72 ± 0.23^{bc}	1.89 ± 0.24^{bc}	1.65 ± 0.21^{bc}	1.81 ± 0.24^{bc}	2.05 ± 0.31^{bc}
SP600125 group C	1.45 ± 0.18^{bcd}	1.39 ± 0.22^{bcd}	1.31 ± 0.19^{bcd}	1.52 ± 0.21^{bcd}	1.62 ± 0.24^{bcd}

β-actin was used as internal reference to calculate the protein expressions of Bax, CytC, Fas, FasL and Caspase-3. [a]: $P < 0.05$ compared with control group; [b]: $P < 0.05$ compared with 40 μmol/L juglone group; [c]: $P < 0.05$ compared with SP600125 group A; [d]: $P < 0.05$ compared with SP600125 group B.

molecules is a way to activate the JNK/c-Jun pathway. In order to clarify the role of JNK/c-Jun pathway in the juglone-inducing apoptosis of cervical cancer cells, the expression of p-JNK and p-c-Jun in Hela cells treated with juglone was analyzed in the present study, and the results showed that juglone significantly increased the protein expressions of p-JNK and p-c-Jun ($P < 0.05$), and the protein expressions increased more remarkably as the treatment period and juglone dosage increased ($P < 0.05$), thus indicating that juglone significantly activated JNK/c-Jun signaling pathway in cervical cancer cells and suggesting that juglone mediated apoptosis by activating JNK/c-Jun pathway.

In order to confirm the speculation that juglone may mediate apoptosis through JNK/c-Jun pathway, JNK-specific inhibitor SP600125 combined with juglone was used to treat cervical cancer cells. The role of JNK in the juglone-inducing apoptosis of cervical cancer cells was confirmed by comparing with the juglone treatment alone. The expression of apoptotic molecule of intracellular mitochondrial pathway and death receptor pathway was analyzed, and the results showed that the protein expressions of Bax, CytC, Fas, FasL and Caspase-3 in cells treated with 40 μmol/L juglone + different dosages of SP600125 were significantly lower than those in cells treated with 40 μmol/L juglone alone ($P < 0.05$), and the expressions reduced more significantly as the dosages of SP600125 increased ($P < 0.05$). This suggests that the effect of juglone on the protein expression of Bax, CytC, Fas, FasL and Caspase-3 in cervical cancer cells were reversed by JNK inhibitor SP600125, thus confirming that juglone regulated expression of apoptotic molecules of mitochondrial pathway and death receptor pathway in cervical cancer cells through JNK/c-Jun signaling pathway, and induced apoptosis of cervical cancer cells.

In conclusion, juglone can significantly induce apoptosis of cervical cancer cells; activating JNK/c-Jun pro-apoptotic pathway and increasing expression of apoptotic molecules in downstream mitochondrial pathway and death receptor pathway are the molecular mechanisms for juglone to play a pro-apoptotic role.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

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