· 药理实验与临床观察 ·

Organic extract of *Tripterygium hypoglaucum* induced apoptosis of HL-60 cells through NF-κB and mitochondrial signaling pathways as revealed by cDNA microarray technique

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Abstract **Object** To investigate the signaling pathways and molecular mechanism involved in the apoptosis of human promyleocytic leukemia HL-60 cells induced by organic extract of *Tripterygium hypoglaucum* (THH) root. **Methods** Cell biological changes during the apoptosis induction were studied by flow cytometry. Molecular mechanism involved in the apoptosis was examined by cDNA microarray technique. **Results** Flow cytometric study confirmed the induction of apoptosis by the organic extract of THH. The gene expression profiles of HL-60 cells treated by THH were obtained using a cDNA microarray containing 3 000 human genes derived from a leukocyte cDNA library. Sixteen genes identified to be differentially expressed in HL-60 cells can be clustered into groups related to apoptosis (including Caspases 3 and Caspase 8), cell proliferation, cell cycle control and differentiation, and stress response. **Conclusion** The induced apoptosis of HL-60 cells by THH extract is associated with the up-regulation of several genes (such as NFKBIB, PRG1 and B2M) related to NF-κB and mitochondrial signaling pathways.

Key words *Tripterygium hypoglaucum* (Lévl.) Lévl. ex Hutch.; apoptosis of HL-60 cells; mitochondrial signal

基因芯片分析显示昆明山海棠有机萃取液经 NF-κB及线粒体信号传导途径诱导 HL-60细胞凋亡

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摘 要:目的 研究中草药昆明山海棠 *Tripterygium hypoglaucum* (THH)有机萃取液诱导人早幼粒白血病 HL-60细胞凋亡过程的信号传导通道及分子机制。方法 应用流式细胞仪研究了 THH有机萃取液诱导 HL-60细胞的凋亡过程,并应用包含 3 000人类基因与 EST的基因芯片进行基因表达差异分析。结果 基因芯片杂交结果显示有 16个基因表达发生大于 2倍的显著变化,这些基因同细胞生长,细胞周期调控,细胞分化,以及压力反应有关。部分基因在细胞凋亡过程中起关键作用,如 Caspase 3和 Caspase 8 结论 THH有机萃取液诱导 HL-60细胞凋亡,该过程与 NF-κB和线粒体信号传导途径有关。

关键词:昆明山海棠; HL-60细胞凋亡; 线粒体信号

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Introduction

The plants of *Tripterygium* Hook. f. (Celastraceae) have been used in traditional Chinese medicine as remedies for cancer treatment and as

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an insecticide for hundreds of years[1-4]. Our previous study has shown that the root extract of *Tripterygium hypoglaucum* (Lévl.) Lévl. ex Hutch. (THH) can induce apoptosis in leukemia cell line HL-60 cells and alter the expression pattern of c-Myc gene. The purpose of this study is to examine whether the organic extracts of THH may induce apoptosis in HL-60 cells also and to further investigate the molecular mechanism of such induction process.

In the present study, flow cytometry and cDNA microarray techniques were used to investigate the cellular and molecular effects of THH organic extract on HL-60 cells. Flow cytometry has been used to detect and quantitate various aspects of cell death, including apoptotic or necrotic cells[5]. Apoptotic cells contain reduced DNA content and will appear as cells with low DNA stainability ("sub-G1" peak) as compared to that of G1 cells[6]. cDNA microarray technique allows monitoring of the expression of hundreds and thousands of genes simultaneously[7,8]. In this study, a cDNA microarray containing 3 000 human genes derived from a leukocyte cDNA library was used to investigate the gene expression profiles of HL-60 cells upon THH treatment.

Materials and methods

1. THH preparation and cell culture. Organic extract of THH was prepared according to the process shown in Fig. 1. The extract was stored at dimethylsulfoxide (DMSO) as a concentration of 20 μg/μL. HL-60 cells (American Type Culture Collection, MD, USA) were grown at 37 ℃ in 5% CO_2 in RPMI-1640 supplemented with 1% antibiotic solution (Invitrogen) and 10% heat-inactivated fetal bovine serum (Invitrogen). For THH treatment, 2×10^5 cells/mL were used and treated with 25 μg/mL or 40 μg/mL of THH extracts for 8 hours.

2. Flow cytometry study. the control or THH-treated cells $2-3\times10^6$ were collected by centrifugation and washed twice by phosphate-buffered saline (PBS). The cell pellets were resuspended gently in 1 mL of hypotonic propidium iodide solution (50 μg/mL) prepared in 0.1% sodium citrate plus 0.1% Triton X-100 and 100 mg/mL DNase-free RNase A for DNA staining. Stained cells were analyzed by ESP flow cytometry (Coulter Electronic, USA) at excitation 488/emmision 600 nm. Data were analyzed by cycle distribution software (ModFit LT version 2.0, Verity Software House, USA).

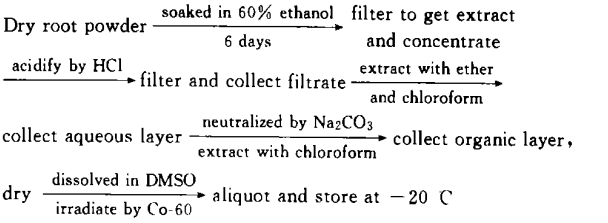


Fig. 1 Process for preparation of organic extract of THH

3. Total RNA preparation and fluorescence labeling. HL-60 cells (control) and treated with THH (40 μg/mL, 8 h) were harvested and collected in a 50 mL conical tube by centrifugation (1 000 r/min, 5 min). Cells were lysated and total RNA isolated with TRIZOL reagent (Invitrogen). The concentration of total RNA was measured with a biophotometer, and 500 ng of each total RNA sample was used to run a 1% denatured agarose gel to verify quality. Same amount of total RNA control or treated total RNA were reverse transcripted to cDNA in the presence of two distinct fluorescent dyes, Cy3-dUTP and Cy5-dUTP, respectively. The labeled cDNA was purified using Microcon 30 (Millipore).

4. cDNA microarray hybridization. A cDNA microarray was prepared by using 3 000 cDNA probes amplified from a leukocyte cDNA library (clontech) and arrayed on glass slides using a microarrayer (SPBIO, Hitachi). The labeled cDNAs were mixed and denatured at 100 ℃ for 2 minutes and hybridized with the microarray at 65 ℃ overnight. Microarray images were obtained by a confocal fluorescence scanner (ScanArray 4 000, GSI Lumonics, USA) and the scan results were analyzed using ScanAnalyzer software (Standford University, California, USA). Fluorescence ratios (Cy5 vs. Cy3) were used to determine the differential gene expression levels. Genes with ratio

number above 2 or under 0.5, i.e. induction or repression of 2 fold, were selected and subjected to further analysis.

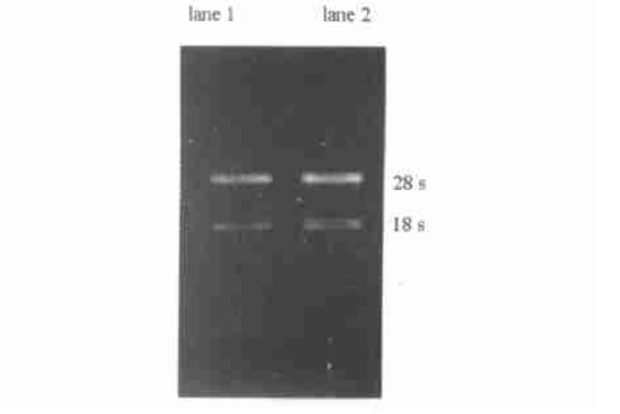
Result and discussion

1. Organic extract of THH induced apoptosis in HL-60 cells. Flow cytometry was used to study the effect of THH organic extract on HL-60 cells. Cells were treated with 0 (control), 25 and 40 μg/mL of THH extract for 8 hours. The proportion of sub-G1 cells increased by 15 to 45 folds after 25 and 40 μg/mL treatment, respectively, as compared to the untreated cells (Table 1). It has been shown that the accumulation of dying cells in this "sub-G1" hypodiploid peak is mainly due to a reduced DNA content and these cells are destined to enter apoptosis[5]. The data confirmed that HL-60 cells were induced to enter apoptosis after THH treatment.

Table 1 Proportion of sub-G1 phase in HL-60 cells after 8 h THH treatment

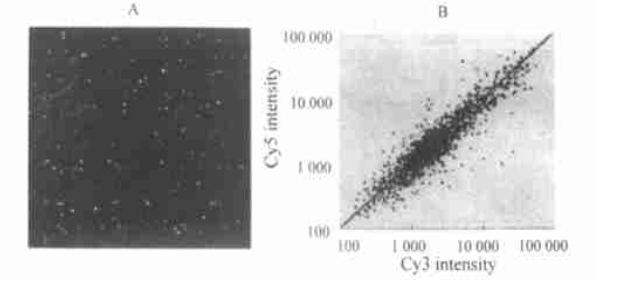
Treatment dose/(μg·mL^{-1})	Proportion of sub-G1/%
0	0.2
25	3.1
40	9.3

2. THH treatment changed gene expression profiles of HL-60 cells. Total RNA from untreated and THH-treated HL-60 cells were used as templates for synthesis of Cy3 (green) and Cy5 (red) labeled cDNA probes, respectively. The quality of the total RNA was verified by gel electrophoresis (Fig. 2). Fig. 3A showed a typical microarray image following the competitive hybridization of the two labeled cDNAs with the microarray containing 3 000 probes. A scattered plot of Cy5 intensity versus Cy3 intensity showed the quality of hybridization to be reasonable without obvious bias (Fig. 3B). After preliminary data analysis, 16 genes which displayed a greater than 2-fold difference between untreated and drug treated samples were identified. The accession numbers, functional description, as well as the Cy5/Cy3 fluorescence ratios of these genes were listed in Table 2. The sixteen differentially expressed genes can be classified into three main categories: genes related to apoptosis, genes related to cell cycle and differentiation, and genes related to stress response (Table 2). Both Caspases 3 and Caspase 8, important common genes in executing the apoptosis process, were upregulated, which is consistent with the observation by flow cytometry that HL-60 cells entered active apoptosis process following the THH treatment.



500 ng of total RNA isolated from control (lane 1) and THH-treated (40 μg/mL, 8 h, lane 2) HL-60 cells were electrophoresed on a 1% formaldehyde denatured agarose gel

Fig. 2 Quality of total RNA isolated from HL-60 cells



(A) Total RNA were extracted from both control and treated cells (40 μg/mL of THH with 8 h) and reverse transcribed to cDNA with Cy3 (green) and Cy5 (red) labeled, respectively. Labeled cDNA were mixed and hybridized to a microarray. Image was obtained using a confocal fluorescence laser scanner (GSI 4000). (B) Scattered plot of Cy5 intensity versus Cy3 intensity.

Fig. 3 cDNA microarray image showing gene expression profiles of HL-60 cells

3. THH-induced apoptosis involved NF-κB and mitochondial pathways. Among the apoptosis related genes, most are involved in two signaling pathways leading to apoptosis, including NF-κB signaling pathway and mitochondrial mediated signaling pathway. For example, NFKBIB, the gene encoding the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor beta, was up-

Table 2 Differentially expressed genes in HL-60 cells following THH treatment

Category	Description of gene	Accession Number	Fold
Apoptosis	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB)	AI935157	4.35
	proteoglycan 1, secretory granule (PRG1)	NM 002727	3.76
	Caspase 8, apoptosis-related cysteine protease	BG529842	3.15
	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	BG491863	3.08
	Capase 3, apoptosis-related cysteine protease	AU125557	2.86
	beta-2-microglobulin (BM)	AV710740	2.72
	ATP synthase, H+ transporting, mitochondria F0 complex, subunit g	AV714814	2.63
	mitochondrial ribosomal protein S12	AF058761	2.56
	non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	AL043778	0.47
	c-myc binding protein	AL561551	0.33
Cell cycle and differentiation	HIR histone cell cycle regulation defective homolog A (*S. cerevisiae*)	NM_003325	2.18
	ribosomal protein L31	AW973154	2.56
	interleukin 10 receptor, beta	BC001903	0.48
	cyclin B2	N87720	0.32
Stress response	heat shock 7× 10^4 protein 4	BE742483	2.05
	heat shock 9× 10^4 protein 1, beta	BG336532	2.13

regulated by more than 4-fold. NFKBIB is the central gene in the NF-κB signaling regulation[9,10]. Proteoglycan 1, secretory granule (PRG1), an early response gene in pancreatic cancer cell regulated by P53 and NF-κB[9], was up-regulated by more than 3-fold. Beta-2 microglobulin (B2M) as the NF-κB target gene for immunoreceptor[9] was also up-regulated during THH treatment. On the other hand, the up-regulation of ATP syntheses, solute carrier family (mitochondrial carrier; phosphate carrier) member 3, and mitochondrial ribosomal protein S12, indicates the involvement of the mitochondrial mediated apoptotic pathway. It was well known that apoptosis is an active, ATP-requiring process[11]. Two genes were down regulated, including an NDP kinase protein expressed in non-metastatic cells 5, and cmyc binding protein. Both are involved in the proliferation of cells and it is expected that their activities need to be attenuated during apoptosis[11].

Several cell cycle related genes, such as cyclin B2 and interleukin 10 receptor were down regulated after THH treatment, consisted with the fact that the HL-60 cells were induced into apoptosis. Two genes related to cell differentiation, e.g. ribosomal protein L31 and a histone family gene were up regulated, which showed that THH extracts may also induce HL-60 cell differentiation. In addition, several stress response genes, including heat shock 7× 10^4 protein 4 and 9× 10^4 protein 1, beta, were up regulated during THH treatment, which is a common response mechanism for cells under drug treatment.

In conclusion, floe cytometric study and the up-regulation of Caspases 3 and Caspase 8 as revealed by cDNA microarray study has demonstrated that HL-60 cells entered active apoptosis process following the THH extract treatment. Several genes related to cell apoptosis were up-regulated, mainly involved in NF-κB and mitochondrial signaling pathways.

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草问荆总生物碱对大鼠脑内氨基酸类神经递质含量和纹状体内乙酰胆碱含量的影响

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摘 要: 目的 研究草问荆总生物碱 (TAEP) 对大鼠脑内氨基酸类神经递质含量和纹状体内乙酰胆碱 (Ach) 含量的影响, 揭示其对中枢神经系统抑制作用的作用机制。方法 采用双波长扫描定量法和豚鼠回肠生物测定法观察大鼠脑内氨基酸类神经递质的含量和纹状体内 Ach含量。结果 TAEP对大鼠脑内 4种氨基酸类神经递质 (谷氨酸、甘氨酸、γ-氨基丁酸、天冬氨酸) 的含量均无影响, 但可显著降低大鼠纹状体内 Ach的含量。结论 TAEP对中枢神经系统的抑制作用与脑内 4种氨基酸类神经递质 (谷氨酸、甘氨酸、γ-氨基丁酸、天冬氨酸) 的含量无关, 而是通过降低 Ach 的含量, 进而影响多巴胺-2 (DA-2) 受体达到的。

关键词: 草问荆总生物碱; 氨基酸类神经递质; 乙酰胆碱

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Effect of total alkaloids of *Equisetum pratense* on amino acid neurotransmitters and Ach of striatum in rat brain

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Abstract **Object** To study the effect of total alkaloids of *Equisetum pratense* Ehrh. (TAEP) on the contents of amino acid neurotransmitters and Ach in rat brain to reveal the mechanism of TAEP inhibitory action on the central nervous system (CNS). **Methods** Contents of amino acid neurotransmitters and Ach in rat brain were determined by double-wavelengh scan and GPI mensuration. **Results** TAEP could not influence four kinds of content of amino acid neurotransmitter (glutamic acid, glycin, γ-aminobutyric acid, aspartic acid), but TAEP could significantly lower the content of Ach in striatum of rat. **Conclusion** The inhibition of TAEP to CNS is attained by lowering the content of Ach in striatum to affect DA-2 receptor, and it is irrelevant to the amino acid neurotransmitters (glutamic acid, glycin, γ-aminobutyric acid, aspartic acid).

Key words total alkaloids of *Equisetum pratense* Ehrh. (TAEP); amino acid neurotransmitters; Ach

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