· 药理实验与临床观察 ·

Apoptosis in HL-60 cells induced and c-Myc expression down-regulated by root aqueous extract from *Tripterygium hypoglaucum*

FONG Wang-fun[1], ZHUANG Wei-jian[1], CAO Jia[2], LEUNG Chung-hang[1], CHEUNG Hon-yeung[1], XIAO Pei-gen[3], YANG Meng-su[1,4*]

(1. Bioactive Products Research Group, Department of Biology & Chemistry, City University of Hong Kong, Kowloon, Hong Kong, China; 2. Molecular Toxicology Laboratory, Third Military Medical University, Chongqing 400038, China; 3. Institute of Medicinal Plants, CAMS& PUMC, Beijing 100094, China; 4. Applied Research Centre for Genomics Technology, City University of Hong Kong, Kowloon, Hong Kong, China)

Abstract **Object** To investigate the effects of the root aqueous extract of *Tripterygium hypoglaucum* (Levl.) Hutch (Celastraceae) (THH) on human promyelocytic leukemia HL-60 cells and its mechanism. **Methods** Cell growth and toxicity assays, flow cytometry, confocal fluorescence microscopy and Annexin-V labeling, and Western Blotting Assay were used. **Results** THH caused morphological changes hypodiploid sub-G1 cell accumulation and induced dose- and time-dependent apoptosis in HL-60 cells at concentrations above 18 μg/mL. Western Blotting analysis showed a more than 99% decrease of the nuclear oncoprotein c-Myc after 2~4 h of THH treatment. **Conclusion** THH decreases c-Myc protein expression, leading to the deactivation of cell cycle progression pathways and an accumulation of hypodiploid sub-G1 cells that eventually enter apoptosis.

Key words *Tripterygium hypoglaucum* (Levl.) Hutch. (Celastraceae) (THH); HL-60 cells; apoptosis; c-Myc protein expression

昆明山海棠根部水提液诱导 HL-60细胞凋亡及对 c-Myc基因表达的调控

方宏勋[1*],庄为筧[1],曹 佳[2],梁重恒[1],张汉扬[1],萧培根[3],杨梦苏[1,4*]

(1. 香港城市大学 生物及化学系生物活性制品研究组,香港 九龙; 2. 中国人民解放军第三军医大学 分子毒理学实验室,重庆 400038; 3. 中国医学科学院 中国协和医科大学药用植物研究所,北京 100094; 4. 香港城市大学 基因组科技应用研究中心,香港 九龙)

摘 要: 目的 研究昆明山海棠 *Tripterygium hypoglaucum* (Celastraceae) (THH) 根部水提液对早幼粒白血病 HL-60细胞凋亡的诱导作用及机制。方法 通过特征性的形态学观察, Annexin-V 标记,以及流式细胞仪检测中次 G_1峰的形成确定 THH的诱导细胞凋亡作用;应用 Western Blotting 研究 THH对 c-Myc蛋白表达的影响。结果 THH根部水提液可诱导早幼粒白血病 HL-60细胞凋亡。在浓度高于 18 μg/mL时, THH对 HL-60细胞的诱导凋亡作用呈现浓度与时间的相关性。在 THH处理 2~4 h后,癌基因蛋白 c-Myc表达降低 99% 以上。结论 THH抑制了 c-Myc蛋白的翻译或后翻译过程,从而降低了 c-Myc在细胞周期运转中的作用,导致大量细胞凋亡。

关键词: 昆明山海棠; HL-60细胞; 凋亡; c-Myc蛋白表达

中图分类号: R285.5 文献标识码: A 文章编号: 0253-2670(2003)07-0622-04

Introduction

The root of *Tripterygium hypoglaucum* (Levl.) Hutch. (THH), a well-known and readily available Chinese medicinal product, has been used for the treatment of auto-immune diseases including rheumatoid arthritis, systemic lupus erythematosus (SLE) and skin problems. A number of preliminary studies have suggested the possibility that THH affect T lymphocytes[1] and may induce chromosome changes in certain cells[2]. It was suggested that THH may cause apoptosis in a number of cultured tumor cells, with leukemic cells being the most sensitive[3-5]. However, the effects of THH-induced apoptosis and its action mechanism have not been investigated in detail.

The objective of the present study is to exam-

* 收稿日期: 2002-10-02
收稿日期: 方宏勋,男,香港城市大学生物及化学系教授,主要研究方向: 肿瘤细胞的生化及分子生物学、生物活性制品和中草药药理学。
E-mail bhwffong@cityu.edu.hk

ine the effect of THH treatment on the apoptosis in human promyelocytic leukemia HL-60 cell line. In addition, because the nuclear oncoprotein and transcriptional activator c-Myc is one of the central players in regulating cell proliferation, differentiation and apoptosis[6,7], the expression of c-Myc protein was also determined in order to provide insights into the molecular mechanisms involved in THH-induced apoptosis.

Materials and methods

Preparation of THH extract. Dried root of *T. hypoglaucum* (Levl.) Hutch. (THH) (Yunnan Medicine Company, Kunming) was powdered and soaked in double distilled water (100 g/500 mL) for 24 hours, then boiled and cooled three times. Extract was filtered and concentrated by gentle boiling. After centrifugation, supernatant was filtered by 0.22 μm nitrocellulose membrane and was kept at − 20 ℃.

Cell culture and MTT cytotoxicity assay. HL-60 cells (American Type Culture Collection, MD, USA) were routinely grown at 37 ℃ in 5% CO_2/air in RPMI-1640 supplemented with 1% antibiotic solution and 10% heat-inactivated fetal calf serum (Gibco, MD, USA). For toxicity assay, 1×10^4 cells/well were grown for 12-15 hours in 96-well culture plates and different amounts of THH extract were added, followed by MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma; 5 mg/mL in PBS] as described in the literature[8].

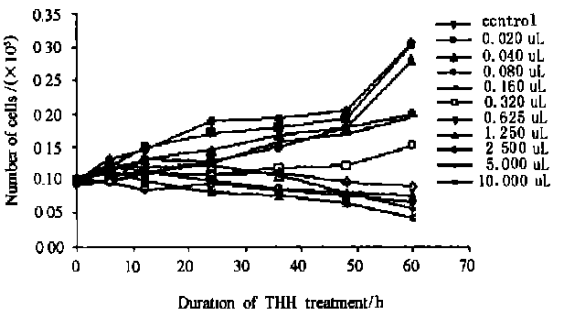
Flow cytometry and confocal microscopy. For flow cytometric measurements, 2×10^6— 3×10^6 cells were washed in phosphate-buffered saline (PBS) and re-suspended in 100 μL fresh medium. Cell suspensions were stored at − 20 ℃ in adding 1 mL 70% ethanol. Cells were collected by centrifugation and incubated with propidium iodide and RNase A. Stained cells were analyzed by EPICS Elite ESP flow cytometer (Coulter Electronic, USA). For confocal microscopic measurements, cells were fluorescence labeled by adding 40 μL staining solution (100 μg/mL each of acridine orange and ethidium bromide) to 1 mL cell suspension or were stained with Annexin-V and propidium iodide (Clontech, MA, USA). The samples were studied with a LSM510 (Carl Zeiss) confocal laser-scanning microscope.

Western Immuno-Blotting. Whole cell extracts containing 15— 20 μg protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking by Tris-buffer saline containing 5% non-fat milk powder, filters were incubated with mouse antihuman c-Myc monoclonal antibody Ab-3 (1∶80 dilution) (Calbiochem, USA), and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibodies (Bio-Rad, USA). Tagged protein bands were detected by treatment with ECL Western Blotting reagents (Amersham, UK) and exposed on X-ray films (Fuji, Japan).

Results and discussion

THH-induced apoptosis in HL-60 cells. Cell growth was inhibited when exposed to THH extract at concentrations above 0.625 μL extract (18.3 μg solid per mL of growth medium) (Fig. 1). The percentage of death cells caused by THH treatment was time- and dose-dependent. Subsequent experiments were performed with cells treated with 5 μL of THH extract (146.5 μg solid per mL of medium).

The morphology of cells was examined by flu-



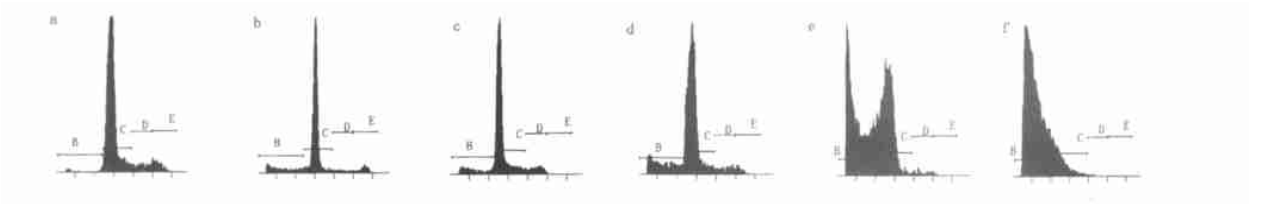
Various amount of THH extract (solid contents 29.3 mg/mL) was added to culture medium and numbers of viable cells were estimated by MTT assay. Data points represent averages of three experiments

Fig. 1 Growth inhibitory effect of THH extract on HL-60 cells

orescence confocal laser scanning microscope where cells were stained with acridine orange/ethidium bromide. Untreated HL-60 cells appeared uniformly green with distinct round nucleoli, while cell shrinkage, chromatin condensation and nuclear fragmentation became obvious in some of the cells after 8 hours of THH treatment. After 12 hours of THH treatment, cellular fragmentation resulted in the appearance of apoptotic bodies (bright orange bleb).

Annexin-V assay was used to probe the redistribution of membrane phosphatidylserine (PS) from cytoplasmic side to outside surface as an early indicator of apoptotic cell death[9]. Annexin-V is a 35.8 kDa protein that has a strong affinity to PS on the outside surface of intact cells. While control cells had little PS on the outside surface, cells treated with THH for more than 2 hours were clearly labeled by Annexin-V (bright green).

Flow cytometric analysis of THH-treated HL-60 cells showed a distinct sub-G1 hypodiploid peak (Fig. 2). The accumulation of dying cells in the "sub-G1" hypodiploid peak is due mainly to a reduced DNA content and these cells are destined to enter apoptosis[10]. The proportion of sub-G1 cells increased with time and 3%, 7%, 13%, 57% and 90% of total cells were in this phase after 2, 4, 8, 12 and 18 hours of treatment, respectively (Fig. 2)

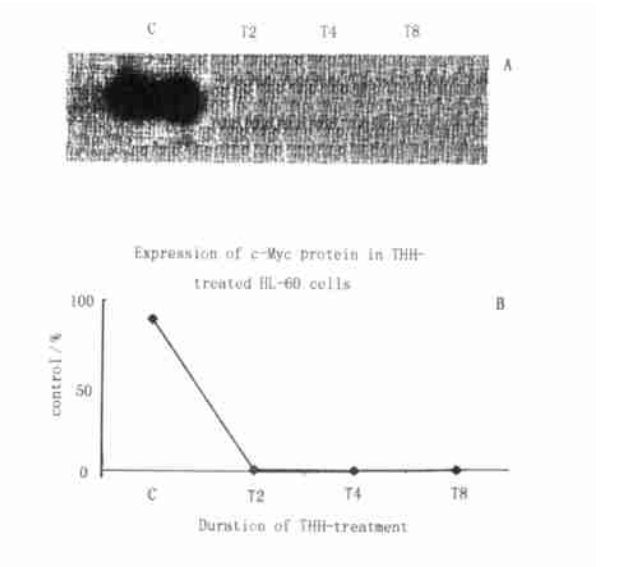


Cells were treated with 5μg/mL THH extract for 0 (control, panel A), 2 (panel B), 4 (panel C), 8 (panel D), 12 (panel E) and 18 (panel F) h. A gradual accumulation of sub-G1 and G1 cells (shown as B zones in graphs) can be seen

Fig. 2 Flow cytometry analyses of THH-treated HL-60 cells

The combination of the cell growth assay, morphological changes, and cell cycle study clearly demonstrated that THH extract caused apoptosis in HL-60 cells. Characteristic apoptotic features were induced at low concentrations of THH extract (above 18 μg/mL) and cells responded after a short exposure time and in a dose- and time-dependent manner.

Down-regulation of c-Myc. Since c-Myc protein played a central role in regulation cell proliferation, differentiation and apoptosis, the expression of c-Myc was measured with Western Immuno-Blotting. In control cells, the expression level of c-Myc remained constant. However, in THH-treated cells, the protein level drastically decreased by 99% after 2- 4 hours of treatment (Fig. 3A, B). The consequences of down-regulating c-Myc were studied in Myc-null cells[11]. The doubling time of these cells was prolonged, particularly when there were accumulations of cells in G1 and G2 phases, whereas S phase was normal. It is conceivable that



Cells were treated with 5μg/mL of THH-extract. Panel A: Western Bloting analyses of c-Myc protein by specific antibodies. Lane c: normal cells; lanes T2, T4 and T8 cells treated with THH extract for 2, 4 and 8 h, respectively. Panel B: graph showing % changes of c-Myc protein. Data are averages of two experiments.

Fig. 3 c-Myc expression

a lowering of c-Myc level could lead to apoptosis by first deactivating the proliferation pathway[12] and preventing cell cycle progression. Treatments that caused apoptotic death of several cell types had been shown to first drastically lower c-Myc expression[13, 14].

While the accumulation of sub-G1 cells and apoptosis took place after 18 hours of THH treatment, c-Myc protein showed a drastic decrease after only a short period of THH treatment (> 99% decrease in 2- 4 hours). This is consistent with the current thinking that c-Myc is one of the earlier signals in cell growth regulation. The above results showed that THH extract initially caused a decrease in c-Myc protein level in HL-60 cells, by either transcriptional or translational regulation mechanisms. Subsequently cellular metabolism is affected[7] and cells stop at the hypodiploid sub-G1 phase and eventually enter apoptosis.

Acknowledgement This work is supported by an Applied Research Grant of City University of Hong Kong.

References

[1] Zhao X Z. Effects of *Astragalus membranaceus* and *Tripterygium hypoglancum* on natural killer cell activity of peripheral blood mononuclear in systemic lupus erythematosus [J]. *Chin J Integrated Tradit Chin West Med* (中国中西医结合杂志), 1992, 12: 669-671.

[2] Wang X, Zhuo R, He Z. Aneuploid induction by water extract from *Tripterygium hypoglaucum* (Levl.) Hutch. in mouse bone marrow cells [J]. *Mutagenesis*, 1993, 8: 395-398.

[3] Cao J, Nusse M. *Tripterygium hypoglaucum* induced apoptosis in Jurkat and two other cell lines [J]. *Chin Sci Bull* (科学通报), 1999, 44(11): 1169-1173.

[4] Kerr J F R, Wyllie A H, Currie A R. Apoptosis: a biological phenomenon with wide-ranging implications in tissue kinetics [J]. *Br J Cancer*, 1972, 26: 239-257.

[5] Jacobson M D, Weil M, Raff M C. Programmed cell death in animal development [J]. *Cell*, 1997, 88: 347-354.

[6] Desbarats L, Schneider A, Muller D, *et al*. Myc: a single gene controls both proliferation and apoptosis in mammalian cells [J]. *Experimentia*, 1996, 52: 1123-1129.

[7] Dang C V. c-Myc target genes involved in cell growth, apoptosis, and metabolism [J]. *Mol Cell Biol*, 1999, 19: 1-11.

[8] Mosmann T. Cytotoxicity assay based on diphenyltetrazolium bromide [J]. *J Immunol Methods*, 1983, 65: 55-63.

[9] Martin S J, Reutelingsperger C P, McGahon A J, *et al*. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl [J]. *J Exp Med*, 1995, 182: 1545-1556.

[10] Darzynkiewicz Z, Li X, Gong J P, *et al*. Analysis of cell death by flow cytometry, in cell growth and apoptosis [M]. (Studzinski GP ed), Oxford: Oxford University Press, 1995.

[11] Sonenshein G E, Down-modulation of c-Myc expression induces apoptosis of B lymphocyte models of tolerance via clonal deletion [J]. *J Immuno*, 1997, 158: 1994-1997.

[12] Prendergast G C. Mechanisms of apoptosis by c-Myc [J]. *Oncogene*, 1999, 18: 2967-2987.

[13] Packham G, Cleveland J L. c-Myc and apoptosis [J]. *Biochim Biophys Acta*, 1995, 1242: 11-28.

[14] Pu L X, Zhang T M. Effects of triptolide on T lymphocyte functions in mice [J]. *Acta Pharmacol Sin* (中国药理学报), 1990, 11: 76-79.

用寡核苷酸芯片研究血虚小鼠造血相关细胞因子基因表达谱

佟 丽[1],陈苏红[1],马增春[1],黄 坚[2], 丁雨[2],王升启[1*]

(1. 军事医学科学院放射医学研究所 全军生物芯片重点实验室,北京 100850; 2. 深圳益生堂生物企业有限公司,广东 深圳 518026)

摘 要:目的 应用细胞因子寡核苷酸表达谱芯片检测血虚小鼠不同脏器组织内造血相关细胞因子差异表达情况,寻找差异表达基因,为血虚证治疗药物机制研究及治疗药物筛选奠定基础。方法 采用 5.5 Gy ^{60}Co-γ 射线照射 Balb/c小鼠,制备血虚模型。在不同时间点提取正常和血虚小鼠不同组织总 RNA,反转录成不同荧光标记的 cDNA探针,与表达谱芯片进行杂交,对扫描数据进行分析获得血虚小鼠造血相关细胞因子基因的差异表达情况。结果 在各组织中共发现 21个差异表达的基因。这些基因的功能大致分为 3类:促细胞生长或增殖,免疫调节,诱导血细胞形成或促造血祖细胞形成集落。结论 照射后上述细胞因子基因的下调,使机体的造血功能下降,造成血虚,证明细胞因子间形成造血调节网络,整体调节机体造血。这与中医的全局理论是一致的。实验证明应用芯片技

* 收稿日期: 2002-09-10
基金项目:全军医药卫生科研基金课题 (01Z019); 北京市科委资助项目 (H010210220113)
作者简介:佟 丽(1974-),女,辽宁人,军事医学科学院放射医学研究所 2000级硕士研究生,研究方向为中药四物汤补血作用机制的基因组学研究。 E-mail: tongli29@sina.com
* 通讯作者 Tel, Fax: (010) 66932211 E-mail: sqwang@nic.bmi.ac.cn