白花蛇舌草激发肿瘤细胞产生超氧化物和激活半胱氨酸天冬氨酸蛋白酶诱导肿瘤细胞凋亡

雅达赋，李少钦
（新加坡国立大学杨潞龄医学院，新加坡 117597）

【摘要】背景与目的：白花蛇舌草是常用于抗肿瘤的中药之一。实验研究和临床使用表明该药有明显的抗肿瘤作用，但是该药抗癌作用的原理尚不清楚。本文研究了白花蛇舌草的乙醇和水的提取物对人急性髓样白血病细胞 HL60 生存和死亡的影响及其机制。方法：我们用白花蛇舌草的提取物处理细胞，在处理后 2 h 开始测量细胞内的超氧化物及各种半胱氨酸天冬氨酸蛋白酶 (caspase) 的水平，并于 24 h 测量细胞的生存及凋亡情况。结果：我们发现乙醇和水的提取物对 HL60 细胞的生存有较强的抑制作用。乙醇提取物能有效地激活 caspase-2 和 caspase-3 并诱发癌细胞凋亡。乙醇提取物在较短的时间 (2 h) 内诱导细胞产生超氧化物。结论：白花蛇舌草乙醇提取物能有效地激发肿瘤细胞产生超氧化物并诱导肿瘤细胞凋亡。我们认为白花蛇舌草的抗癌机制是通过激发肿瘤细胞产生大量超氧化物，致使癌细胞内氧爆破，最终激活凋亡信号网络而使癌细胞凋亡。

【关键词】白花蛇舌草；肿瘤；超氧化物；半胱氨酸天冬氨酸蛋白酶；细胞凋亡


Evidence for Oldenlandia diffusa-evoked cancer cell apoptosis through superoxide burst and caspase activation
Sanjiv Kumar YADAV, Shao Chin LEE
(Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597)

ABSTRACT  Background & objective: Oldenlandia diffusa (Bai Hua She She Cao) is one of the herbs most commonly used in traditional Chinese medicine for treating cancer. Various studies using the herb alone or in combination with other therapy plans have evidenced the effectiveness of the herb in the management of cancers of different tissue origin. However, the mechanisms underlying its anti-cancer activity are unknown. In the present study, we attempted to investigate the apoptotic activity of crude extracts of the herb as well as the possible molecular pathways. Methods: We incubated human promyelocytic leukemia cell line HL60 cells with ethanol or aqueous extracts of the herb, and determined the levels of intracellular superoxide at 2 and 4 hours as well as caspase activity at 3, 6 and 8 hours using photospectrometry. Cancer cell survival and apoptosis were quantified at 24 hours by using MTT and flow cytometry analyses respectively. Results: We found that it dose-dependently inhibited the cancer cell growth in MTT assay. Flow cytometry analysis revealed that it elicited significant production of sub-G1 population of the cells, indicating the extract-evoked cell apoptotic death. The LD50 of the ethanol extract was estimated to be approximately 320 μg/ml. Moreover, treatment of the cancer cells with the ethanol component markedly increased the production of superoxide within few hours. Significant elevation in the protease activities of caspases-2 and -3 were

This study was supported by grants 185-000-085-213, 364-000-053-213 from NM RC and 364-000-061-112 from ARF of Singapore. Correspondence to: Dr. Shao-Chin LEE. National University Medical Institute, Room 02-12, Block MD11, 10, Medical Drive, National University of Singapore, Singapore 117597. Tel: (65)65167635; Fax: (65)67735461. E-mail: nmlsc@nus.edu.sg or lee_shao@hotmail.com
detected at as early as 3 and 6 hours respectively. Conclusion: Our results show that the ethanol extract of the herb effectively evokes cancer cell apoptosis, possibly through burst-mediated caspase activation.

**KEY WORDS**  Oldenlandia diffusa; cancer; superoxide; caspase; apoptosis


1 INTRODUCTION

Oldenlandia diffusa is one of the most commonly used herbs to treat cancers in traditional Chinese medicine (TCM). Clinical and laboratory investigations have repeatedly evidenced the effectiveness of the herb in the management of cancers of different tissue origin. Zhao[1] reported two cases of successful treatment of nasopharyngeal carcinoma using the herb. As usual, the herb was boiled in water, and the supernatant was taken. The two patients were clinically treated with the herb at the doses of 75 g/day for 6 months and 100 g/day for 9 months respectively. At the end of the clinical treatment, cancer tissue regressed to an undetectable state. No recurrence was found in both patients during the follow up of 9 and 6 years respectively, during which they were advised to continue to take the herbal drink. No significant adverse effect was noted during the clinical treatment and follow up. Li and Huang[2] treated 53 patients with non-small cell lung carcinoma using Oldenlandia diffusa extract in combination with recommended chemotherapy, and 33 patients using recommended chemotherapy alone. They found the combination plan produced better treatment outcomes, more effective, less side-effects and better life quality. Similar outcomes of the combinational therapy plan (Oldenlandia extract plus recommended chemotherapy) were noted by Zhang and coworkers[3] in a cohort of 36 patients with the liver cancer and by Huang et al[4] in 40 patients with acute non-lymphocyte leukemia. More recent clinical investigations have also shown satisfactory treatment outcomes using the herb on various types of cancers, mostly in late stage of disease development[5].

In keeping with the clinical observations, the anti-cancer effect of the herb was also noted in studies by using cell line cells and animal models. Shan et al[6] reported that aqueous extract of the herb enhanced the killing power of natural killer cells against cancer cells as well as the phagocytes power of macrophages towards cancer cells. The cancer cell growth inhibitory effect of aqueous fraction of the herb was also observed in more recent in vivo and in vitro studies[7-10]. In the present study, we investigated the effects of crude extracts of the herb on the growth and death of human promyelocytic leukemia cell line HL-60 cells. We also attempted to look into the underlying molecular mechanisms.

2 MATERIALS AND METHODS

2.1 Chemicals and herbal extraction

All chemicals were used from Sigma. Caspase substrates were provided by R&D Systems. Oldenlandia diffusa dried herb was purchased from Eu Yan Sang TCM which is an internationally recognized TCM herbal shop. Oldenlandia diffusa herb was excised into around 0.5 cm in length and mixed with 80% ethanol overnight with gentle shaking at room temperature. The herb residues were then collected through filtering through Whatman paper, and the ethanol extract was dried by using a freeze dryer, weighted, then dissolved in 80% ethanol again for use. Its stock concentration was 60 μg/μl. The residues of the ethanol extraction were used for aqueous extraction, which was performed for 24 hours at room temperature. Again, extract was filtered, vacuum dried and re-dissolved in water at the concentration of 70 μg/μl.

2.2 Cell culture

HL60 cells from American Type Culture Collection (ATCC) were cultured in a humidified CO2 incubation. For herbal treatment, desired amount of ethanol extract was added into cell culture for desired time; wherever possible, an equal volume of 80% ethanol was added into control cultures.

2.3 MTT assay and flow cytometry analysis

For MTT assay, 50 μg of MTT was added to cell culture for 4 hours. Elution of the precipitate was performed with 200 μl of dimethyl sulphoxide (DMSO) plus 10 μl of Tris-glycine buffer (0.1 mol/L Tris, 0.1 mol/L glycine, pH 10.5, with 1 N NaOH). Cell viability was calculated from OD570 by using an ELISA reader. Apoptosis was evidenced and quantified by using propidium iodide (PI) staining for DNA fragmentation followed by
flow cytometry analysis. Cells were harvested in a 15-ml tube, fixed with 70% ethanol, and stained with PI. At least 10,000 events were analyzed by flow cytometry with the excitation set at 488 nm and emission at 610 nm. Data were analyzed by using the software package WinMDI.

2.4 Measurement of superoxide and caspase activity

The lucigenin-based chemiluminescence assay was performed for quantifying the level of superoxide, which was monitored for 60 s in a TD-20/20 luminometer. For caspase activity assay, cell lysates were mixed with reaction buffer (10 mM HEPES, 2 mM EDTA, 10 mM KCl, 1.5 mM MgCl, 10 mM DTT) on ice for 10 min followed by introduction of fluorogenic caspase specific substrate. After 30 min of incubation at 37 °C, the protease activity was determined by measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a spectrofluorometer. Results are shown as x-fold increase, as compared to controls.

2.5 Statistical analysis

Data of continuous variables are expressed as mean ± SD. Multiple comparisons were performed by ANOVA by using the computer software package SPSS. A P < 0.05 was considered as statistically significant.

3 RESULTS

3.1 Ethanol and aqueous extract of Oldenlandia diffusa inhibited HL60 cell survival and elicited the cells apoptosis

As shown in figure 1, after 24 hours of treatment with either ethanol or aqueous extract of the herb, the number of viable cells decreased upon increasing concentrations of extracts, indicating a dose-dependent effect of the extracts on cell growth and viability (Figure 1A and 1B). The LD50 values were estimated to be around 320 and 370 μg/ml for the ethanol and aqueous extracts respectively. Using flow cytometry analysis, sub-G1 fraction was increased upon treatment with the ethanol fraction. Higher concentration of the extract elicited higher percentage of sub-G1 cells, indicating a dose-dependent effect (Figure 1C). The samples treated with the aqueous fraction were not analyzed by flow cytometry.

3.2 Ethanol extract evoked superoxide production

To investigate the signaling pathways underlying the herbal extract-evoked apoptosis, we firstly looked at the cellular redox status by measuring the levels of superoxide. Within 2 hours of treatment (320 μg/ml), the extract largely increased the cellular level of superoxide, for approximately 2 folds. At 4 hours,
Further increment was noted, to around 3 folds, as compared to that in the control cells (Figure 2). The data indicated that the ethanol causes acute superoxide burst in the cancer cells.

3.3 Ethanol extract activated caspase-2 and -3

Since caspase activation is one of the typical features of apoptosis, we then scanned the activities of caspases-2, -3, -6, -7, -8 and -9 in the control and treated (320 μg/ml) samples. No significant increase in the protease activities of caspases-6, -7, -8 and -9 was evidenced. However, a significant and dose-dependent increase in the protease activity of caspase-2 was noted. At 3, 6 and 8 hours of treatment, caspase-2 activity was upregulated for 1.7, 2.7 and 3.3 folds respectively (Figure 3). Caspase-3 activity was also upregulated, but seemed to occur at later time points; the caspase-3 activity started to increase at 6 hours (Figure 3). Activation of caspases further evidenced that the extract could evoke apoptosis of HL60 cells, as activation of caspases in particular caspase-3 is one of the typical features of apoptosis.

4 DISCUSSION

Although the anti-cancer activity of Oldenlandia diffusa has been recognized for long, how it acts on cancer cells remains unknown. A previous report suggested that the herb might stimulate immune cells to kill cancer cells[1], suggesting an indirect effect. Yu and coworkers[11] showed that aqueous extract of the herb could inhibit cancer cell growth and colonigenicity in a dose-dependent manner, with electronic microscopic evidence for plasma membrane damage but no typical features of apoptosis, suggesting a non-apoptotic mechanism. However, Wang and coworkers[11] observed that aqueous extract of the herb evoked cell cycle arrest and apoptotic death of glioma cells. In keeping this, we found that our ethanol extract elicited HL60 cancer cell apoptosis, as evidenced by production of sub-G0 cells and activation of caspases (in particular caspase-3). Thus, Oldenlandia can effectively elicit cancer cell apoptotic death. This supports the ethanol or organic solvent extract of the herb for clinical anti-cancer use[12]. The active component that evokes cancer cells apoptosis remains to be identified; the available experimental data suggest that chemical compounds[15], polysaccharides[15] and proteins[15] are candidates. The aqueous fraction of the herb has similar inhibitory effect on the cancer cell survival (Figure 1B), supporting the traditional method in using the herb by boiling in water. However, further analyses are required to test whether it inhibits cancer cell survival through eliciting apoptosis.

The molecular mechanisms underlying the anti-cancer effect of Oldenlandia diffusa remain to be explored. The herb might contain component(s) that can inhibit mutagenesis, therefore inhibit carcinogenesis[15]. Gao and coworkers[16] reported that the herb had potent regulatory power on calcium metabolism; it dramatically increased intracellular free calcium concentration in Hela cells when added into cell culture medium. This elicits the speculation that it may evoke cancer cell death through calcium signaling. Despite that Oldenlandia diffusa constitutively contains antioxidants, our data showed that it evoked acute and large scale production of intracellular superoxide, indicating the occurrence of oxidative burst, at least under the experimental conditions. This finding is in keeping with the available data that the herb could stimulate nitric oxide synthase[17] and
Inhibitions of the superoxide production by scavengers or inhibitors is required to test whether the activation of caspases as well as apoptosis is mediated by superoxide.

Further studies are required to delineate the signal transduction pathways of the extract-elicited cancer cell apoptosis. Oxidative stress can activate both extrinsic and intrinsic apoptosis pathways which can be investigated by quantification of Fas (CD95) and mitochondrial potential respectively. Inhibitions of the superoxide production by scavengers or inhibitors (i.e., diphenyleneiodonium) and caspase activations by chemical inhibitors or protein knockdown are necessary experiments to substantiate the functional roles of superoxide and caspases in the extract-evoked apoptosis, and to clarify the relationship between the superoxide production and caspase activation.

In conclusion, our data demonstrate the apoptotic effect of ethanol extract of Oldenlandia diffusa on leukemia HL-60 cells. The data also implicate that the apoptosis is mediated by superoxide burst and/or activation of caspase cascade.

REFERENCES


3. Zhang J, Tang L, Yang Z, et al. Superoxide production in macrophages. Since there is a recognized link between intracellular superoxide production (one of the typical states of oxidative stress) and cell apoptosis, we speculate that our ethanol fraction of Oldenlandia diffusa elicits HL-60 cell apoptotic death through superoxide burst and/or activation of caspase cascade. Further study using superoxide production inhibitors is required to test whether the activation of caspases as well as apoptosis is mediated by superoxide.

4. The data also implicate that our ethanol fraction of Oldenlandia diffusa elicits HL-60 cell apoptotic death through superoxide burst and/or activation of caspase cascade. Further study using superoxide production inhibitors is required to test whether the activation of caspases as well as apoptosis is mediated by superoxide.

5. In conclusion, we show that our data demonstrate the apoptotic effect of ethanol extract of Oldenlandia diffusa on leukemia HL-60 cells. The data also implicate that the apoptosis is mediated by superoxide burst and/or activation of caspase cascade.