Anti-Lymphoma and Immunomodulatory Functions of the Aqueous Extract of *Sophora Tonkinensis* in Mice

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Abstract

*Sophora tonkinensis* (Shan-Dou-Gan), used in Chinese medicine, has received a great deal of attention traditionally to antineoplastic on ascitic carcinoma, sarcoma and cervix carcinoma. In this study, we investigated the influence of the aqueous extract of *Sophora tonkinensis* (STAE) on mouse lymphoma cell lines, MOPC-315 and MPC-11, and lymphocytes for apoptotic analysis. The effect of STAE-fed on T<sub>H1</sub>-type cytokine patterns in mice were determined by ELISA and RT-PCR. We have demonstrated that the CD<sub>50</sub> of STAE for 1x10<sup>6</sup> MOPC-315 cells was 1.07±0.02μg/ml by MTT assay. A dose dependent induction of DNA fragmentation in MOPC-315 cells was treated with STAE using TUNEL assay. Then, data had shown that the IL-2, IFN-γ and TNF-α mRNA and protein expressions of spleen cells of STAE-3days-fed mouse were increased in a dose dependent. In conclusion, our findings suggest that STAE could induce the apoptosis of mouse lymphoma, and contained immune modulators for promoting the anti-lymphoma properties in vivo.

Key words: *Sophora tonkinensis*, apoptosis, IL-2, IFN-γ, TNF-α
Introduction

Apoptosis, a morphologically and biochemically defined form of cell death (Green et al., 1994), plays a role in a wide variety of biologic systems, including tissue homeostasis and regulation of the immune system (Chen et al., 2001; Sandstrom et al., 1993). The process is a highly orchestrated cellular pathway leading to activation of the downstream death machinery. The central mediator and executioner of the death machinery is culminates in caspase-dependent cleavage of a set of regulatory proteins, degradation of cellular DNA, and complete disassembly of the cell (Chu et al., 2002; Chu, 2002).

An effective immune response for preventing cancer diseases is related with the activities of antibody via the complement activation and ADCC (antibody-dependent cell-mediated cytotoxicity) pathway (Correa-Oliveira et al., 1989), macrophage (Ottesen, 1979) and T-cell (Buchanan et al., 1973). The sub-population of CD4+ T-cell is introduced as TH1 and TH2 (Mosmann et al., 1986; Mosmann et al., 1989). It has been established that TH1 subset, including secrete IL-2, INF-γ and TNF-α, can promote the activation of Tc and NK cells to act mainly in helping cell-mediated responses, showing cytotoxicity to tumor and virus-infected cells (Young, 1996).

Sophora tonkinensis (Shan-Dou-Gan), used in Chinese medicine, is commonly used to antineoplastic on ascetic carcinoma, sarcoma and cervix carcinoma in mice, raising the level of peripheral white cells in rabbit, promoting the repair of gastric ulcer, and anti-asthmatic properties(O-Ming, 1999). Beside these traditional functions, newly discovered properties have received a great deal of attention recently including: anti-HBV and anti-entroviruses activity (Ding et al., 2006; Guo et al., 2006), and suppressing the proliferation of HL-60 cells (Deng et al., 2007). In this study, we investigated the influence of the aqueous extract of Sophora tonkinensis (STAE) on mouse lymphoma cell lines, MOPC-315 and MPC-11, and lymphocytes for apoptotic analysis. The immunomodulatory effect of STAE-fed on TH1-type cytokine patterns in mice were determined by ELISA and RT-PCR. Thus, we are going to screen the effect of STAE that could regulate mouse cell-mediated responses and evaluated the anti-tumor property.

Materials and Methods

Aqueous Extraction of Sophora tonkinensis

The roots of Sophora tonkinensis were bought from a Chinese medicine store, named Ching-Juan (Taipei, Taiwan) on June 2005. Fifty g of dried root of Sophora tonkinensis was added to 1000 ml of water in an 100°C heating water bath for 1 hour. The supernatant was evaporated by speed vac. The precipitate was weighted and resolved in PBS, and then stored at 4°C for the following assay.

Animal Treatment

8-10 week-age male mice for Balb/c strain (NTHAC, Taiwan) were fed by STAE ranged from 0, 25, 50 to 100 mg/ml over 3 days. Then, the cells were harvested from the spleen of these mice and used for the following assay. The animal treatment has been approved by the Institutional Animal Care and Use Committee of the Hungkuang University.
Preparation of Cell lines and mouse spleen cells

Mouse lymphoma cell lines, MOPC-315 and MPC-11, and mouse spleen cells were cultured in a complete medium containing RPMI (Invitrogen, SSF, USA) supplemented with a 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Utah), 4 mM L-glutamine (Hyclone, Utah), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone, Utah). The exponential growth cells were used for the following assay.

Proliferation by MTT assay

The viabilities of MOPC-315, MPC-11 and mouse spleen cells (1×10^6/ml) with STAE-treatment in a dose dependent manner (ranging from 0, 0.156...to 10μg/ml) for 72 hours were analyzed by MTT assay (Chu et al., 2002). The cytotoxicity was expressed as the ratio of OD_{590} value of STAE-induced cells to that of solely medium-treated cells.

DNA fragmentation assay for cell death

Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate (dUTP) nick-end-labeling assay (TUNEL) was performed using an ApoAlert™ DNA fragmentation assay kit (Clontech, CA, USA). Briefly, the cells (1×10^6/ml) were treated with STAE ranged from 1.70, 2.55 to 3.40 μg/ml for 72 hours at 37°C in a humidified 5% CO₂ incubator. The following assay was described as Chu and Tseng (2002). The apoptotic cells were monitored based on green fluorescence at 520±20 mm by FACScan (Becton Dickinson, CA, USA) (Chu et al., 2002). Data were collected and equipped with the CELLQuest software.

Specific apoptosis was calculated by subtracting background apoptosis observed in control cells from total apoptosis observed in STAE-treated cells.

STAE induced IL-2, TNF-α, IFN-γ and TGF-β protein productions of mouse spleen cells by ELISA

Samples (1×10^6 cells/ml) were harvested from spleen cells of STAE-fed mouse (from 0, 25, 50 to 100mg/ml for 3 days), and cultured with ConA (1μg/ml) for 2 days at 37°C in a humidified 5% CO₂ incubator. The productions of IL-2, TNF-α, IFN-γ and TGF-β in the culture medium taken from the detected STAE and ConA-stimulated cells were measured by Mouse Cytokine ELISA Kits (R&D, DY. USA). The assays were run using the general ELISA protocol with a sandwich design.

STAE induced IL-2, TNF-α, IFN-γ and TGF-β mRNA expressions of mouse spleen cells by RT-PCR assay

Isolation of RNA

Samples (1×10^7 cells/ml) were harvested from STAE-fed (from 25 to 100mg/ml for 3 days) mouse spleen cells and cultured with ConA (1μg/ml) for 36 hours at 37°C in a humidified 5% CO₂ incubator. The isolation of RNA of the cells was performed as Chu, et al., (2002), and a 2 μl aliquot was used for RNA quantification according to GeneQuant II (Pharmacia, USA).

Synthesis of cDNA

The synthesis of cDNA was performed as Chu and Tseng (2002). The cDNA synthesized from each mRNA sample was subsequently used as the PCR template.
Priming

The primers used for PCR amplification were as follows: IL-2 sense sequence 5'-GACACTTG
GTGCTCTTGCTCA-3' and antisense sequence 5'-TCAATTCTGTGGGCTTGCTTG-3'; IFN-γ
sense sequence 5'-GGCCATGTTCTGAGACATTGAACG-3' and antisense sequence 5'-TCTCAT
AGAGAATTCTGTGGCCTGCTTG-3'; TNF-α sense sequence 5'-TCTCATCAGTCTCTATGGCC-3'
and antisense sequence 5'-GGGAGTAGACGATACAGCAGCACATC-3'; TGF-β sense sequence 5'
-AC CGCAACAACGCAATCTAT-3' and antisense sequence 5'- GAGACGCCAGGAATTGTTGC-3'
; β 2 m sense sequence 5'- T GACCGGCTTGTATGCTATC-3' and antisense sequence 5'
- CAGTGTCAGCGCCAGATTATG-3' (Invitrogen, SFF, USA) (Chopra et al., 1997; Ryan et al., 1998).

PCR amplification

The conditions for amplification were denaturizing at 94°C for 50 sec (5 min for the first
cycle), IL-2, IFN-γ, TNF-α and TGF-β annealing at 55°C for 1 min, β2m annealing at 60°C for 45
sec, and extension at 72°C for 45 sec (5 min for the last cycle) (Chopra et al., 1997; Ryan et al., 1998).
The PCR products were subjected to gel electrophoresis, staining by ethidium bromide, and
an immediate density measurement for each band using a Multilmage™ system (Alpha Innotech,
USA).

Statistical analysis

All data were expressed as mean ± SD, and the differences between groups were assessed using
Student’s t-test. A value of p<0.05 was considered significant.

Results

Viability effect of STAE in mouse lymphoma
and normal spleen cells

A number of biological functions of *Sophora
tonkinensis* have been reported, such as:
immune modulator, antineoplastic and antitumor
activity (O-Ming, 1999). In this study, STAE
were found having an efficiently cytotoxic effect
on MOPC-315 cells, a weaker cytotoxic effect
on MCP-11 cells and resistant completely in the
same condition on mouse spleen cells. The 50%
cytotoxocities of STAE were 1.07±0.02 μg/ml
for 1x10⁶ MOPC-315 cells and 1.65±0.05 μg/ml
for MCP-11 (Fig.1A). This data indicated that
STAE had a good anti-lymphoma activity,
and the subsequent study of DNA fragmentation
was performed for STAE-treated MOPC-315 cells
(Fig.1A).

Apoptotic induction of STAE in MOPC-315
cells

The results of TUNEL analysis revealed that
the extent of DNA fragmentation in 1.70, 2.55
and 3.40 μg/ml of STAE-treated MOPC-315 cells
were significantly increased from 0.75%, 46.88%
to 79.41% , respectively (Fig 1B). Data had shown
that a significant apoptosis was induced in STAE-
treated MOPC-315 cells.

The expressions of IL-2, IFN-γ, TNF-α and
TGF-β mRNA and protein in spleen cells of
STAE-fed mouse

To investigate the involvement of IL-2,
IFN-γ, TNF-α and TGF-β, the protein expression
for STAE-fed mouse spleen cells was assayed
by ELISA, and the level of mRNA expression in the cells was estimated using RT-PCR. Results indicated that 50, 100 mg/day of STAE-fed led to 10.40±0.52% and 17.91±1.06% increases in the IL-2 mRNA expression of ConA-stimulated spleen cells, respectively (Fig.2A). The IFN-γ mRNA expression in ConA-stimulated spleen cells of STAE-fed mouse led to 4.72±0.44%, 6.98 ±0.46% and 12.71±0.93% increases, respectively (Fig.2A). Then, the TNF-α mRNA expression of ConA-stimulated spleen cells of STAE-fed mouse had showed a 5.83±0.24% increase at 50 μg/ml of STAE-fed and reached the peak increase of 13.23±1.72% at 100 μg/ml of STAE-fed (Fig.2A). However, the TGF-β mRNA expression remained as stable as compared to the control under the same condition (Fig.2A).

STAE could enhance significantly the IL-2 and IFN-γ protein secretions of ConA-stimulated spleen cells of STAE-fed mouse (Fig.3A, Fig.3B), little increase the TNF-α protein secretion(Fig.3C), and no significant difference in TGF-β protein expression under the same condition (Fig.3D). Results for the IL-2, IFN-γ, TNF-α and TGF-β protein production of ConA-stimulated spleen cells of STAE-fed mouse gave virtually correlated results to the mRNA expression data as described above.

**Discussion**

Chinese medicine has been used to treat cancers for as long as this disease has been causing suffering to humankind, and alongside conventional medicine. In this paper, we clearly found that the viabilities of MOPC-315 and MPC-11 cells were decreased after STAE-treatment. On the other hand, the viability of mouse spleen cells in the same range of STAE-additions showed no significant differences as compared with the control groups. Data supported that STAE has no toxic properties. Therefore, our findings indicated that STAE could produce an anti-proliferative effect on different types of lymphoma cell lines, and induced a pro-apoptotic effect on MOPC-315 cells that was confirmed by TUNEL assay.

In the other hand, the mRNA and protein expressions of IL-2, IFN-γ and TNF-α from T cell activation in ConA-stimulated spleen cells of STAE-fed mouse were tested. Results showed that the IL-2 and IFN-γ mRNA and protein productions of 50-100 μg/ml STAE-fed spleen cells were dominantly expressed, the mRNA and protein expressions of TNF-α was weakly enhanced, and no significant change occurred in the TGF-β expression. Therefore, how to regulate the activation of T cells, NK cells, B cells and macrophages, and affect the apoptotic ability of STAE-fed mice to lymphoma and others will be interesting.

The roots of *Sophora tonkinensis* contain alkaloids that possess broad biological activities, for example: -(-)14 beta-hydroxyoxymatrine, (+)-sophoranol and (-)-cytisine showed anti-HBV activity (Ding et al., 2006), and tonkinensisol moderated the cytotoxicity of HL-60 cells (Deng et al., 2007). Based on these findings, we postulate that STAE has immunomodulatory potential for the development of an effective strategy against cancer and other diseases. The strategy would likely have no toxic side-effects. Therefore, STAE may be considered a complementary alternative drug
and an immunostimulatory potentiator. Although the precise mechanism for promoting immunity using STAE still remains unclear, our study should provide new insights for further pertinent investigations to confirm and extend the effects in immune responses in vitro and in vivo, for analysis of active ingredients and major components of STAE in terms of the immunomodulatory effects, and for treatment of anti-lymphoma diseases.

Acknowledgments

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References


Figures

(A)

(B)

Fig.1: STAE induced apoptosis in MOPC-315 cells. (A) The cytotoxicities of MOPC-315 and MPC-11 lymphoma cells, and mouse lymphocytes (1×10^6 cells/ml) treated with STAE ranged from 0.156 to 10 μg/ml were harvested after 72 hours and analyzed by MTT assay. Controls were cells treated with the same condition without STAE only. Data are means ± SD of six similar experiments. (B) A dose-dependent induction of DNA fragmentation in MOPC-315 cells treated with STAE. MOPC-315 cells (1×10^6 cells/ml) were treated with STAE ranged from 1.70, 2.55 to 3.40 μg/ml for 72 hours (thick line) and controls were cells treated with culture medium for the same condition (thin line). Experiments were repeated twice and yielded similar results.
Fig. 2: STAE increased the IL-2, IFN-γ, and TNF-α mRNA expressions in spleen cells of mice in a dose-dependent, and no significantly effect in the TGF-β mRNA expression. Spleen cells were from 25, 50, and 100 mg/day STAE-fed mice for 3 days treatment, and a background control was fed with PBS only. The amount of mRNA was estimated using RT-PCR. Experiments were repeated twice and yielded similar results.
Fig. 3: STAE promoted the IL-2, IFN-γ and TNF-α production of mouse spleen cells and showed no significant effect in the TGF-β production. (A) STAE induced the IL-2 secretion of spleen cells of 25, 50 and 100 mg/day STAE-fed mice for 3 days. (B) STAE induced the IFN-γ secretion of spleen cells in the same condition. (C) STAE induced the TNF-α secretion of spleen cells of 50 and 100 mg/day STAE-fed mice for 3 days. (D) The TGF-β secretion of spleen cells was no change as compared to the control. Cells (1×10⁶ cells/ml) were treated with 5 μg/ml ConA for 2 days. The amount of cytokine was analyzed by ELISA assay. Data are the means ±SD of six similar experiments. * p<0.05 indicated a significant difference from untreated cells.
中草藥山豆根對抗老鼠淋巴癌及免疫調節功能之研究

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摘要

山豆根(Sophora tonkinensis；Shan-Dou-Gan)在傳統醫療上對老鼠腹水癌、肉瘤及子宮頸癌等腫瘤具有抑制作用。本研究探討山豆根水溶液（簡稱STAE）對老鼠淋巴癌細胞MOPC-315、MPC-11及老鼠脾臟細胞等細胞的凋亡作用及其免疫調節功能影響。結果顯示：(1)利用MTT試驗分析，STAE對MOPC-315(1×10⁶/ml)的50%胞殺濃度為1.07 ± 0.02 μg/ml。(2)利用TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)試驗及流式細胞儀(Flow cytometer)分析，證明STAE具有誘發MOPC-315老鼠淋巴癌細胞凋亡的能力。(3)連續餵食3天不同濃度STAE（25, 50, and 100mg/天）之老鼠脾臟細胞，進行RT-PCR及酵素免疫分析法(ELISA)檢查，發現STAE能促進IL-2、TNF-α及IFN-γ等T₃₃型細胞激素之mRNA表現與蛋白質的分泌。總而言之，本研究證實老鼠淋巴癌細胞MOPC-315在STAE作用下會發生凋亡現象，藉以達到殺死癌瘤之目的；同時證實STAE具有增強老鼠抗癌免疫力之功效。

關鍵詞：山豆根，細胞凋亡，IL-2，IFN-γ，TNF-α

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