A Chinese Herbal Medicine, Fu-Ling, Regulates Interleukin-10 Production by Murine Spleen Cells

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Abstract: Fu-Ling is one of the most widely used Chinese herbal medicines. In this study, we investigated the regulatory effect of Fu-Ling on interleukin-10 (IL-10) production in vivo. Mice were i.p. administered 0.1 mg to 1.0 mg Fu-Ling per gram body weight daily for three days. The spleen cells were isolated and assayed for both IL-10 and immunoglobulin (Ig) production. Results indicated that the mice treated with Fu-Ling had significantly increased spleen cell ability to secrete IL-10. Spleen cells isolated from the mice injected with either 0.1 mg or 1.0 mg Fu-Ling per gram body weight also showed an increase in IL-10 mRNA expression. As IL-10 is a potent differentiation factor of B-lymphocytes, the possible effect of Fu-Ling on Ig production was also studied. Results indicated that Fu-Ling significantly induced an increase in IgG and IgA secretion by spleen cells but showed no effect on IgM secretion. Thus, Fu-Ling may affect the function of B-lymphocytes via stimulating IL-10 production.

Keywords: Fu-Ling; IL-10; Immunoglobulin.

Introduction

Tonic recipe is one of the major groups of Chinese herbal medicines believed to be highly beneficial to the immune and gastrointestinal systems in chronic disease patients. Although clinical trials have been performed in Asian countries for hundreds of years, detailed pharmacological studies of traditional tonic recipes are still in the early stages. Si-Jun-Zi-Tang is one of the most widely used tonic recipes in Chinese herbal medicine. Si-Jun-Zi-Tang consists of four major herbs, Ren-Shen (Panax ginseng C. A. Mey), Fu-Ling (Poria cocos (Schw.) Wolf), Bai-Zhu (Atractylodes macrocephala Koidzumi) and Gan-Cao (Glycyrrhiza uralensis Fischer). Our previous studies demonstrated that Si-Jun-Zi-Tang
regulated immunoglobulin A (IgA) production by human peripheral blood mononuclear cells under in vitro conditions (Lu et al., 1994). Si-Jun-Zi-Tang enhanced granulocyte macrophage colony-stimulating factor (GM-CSF) secretion by human peripheral blood mononuclear cells (Tseng and Li, 1996). Among the components in Si-Jun-Zi-Tang, Fu-Ling may be the active ingredient that stimulates IgA production and augments GM-CSF secretion. Fu-Ling significantly enhanced the secretion of interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), but suppressed the secretion of transforming growth factor-β (TGF-β) in vitro (Yu and Tseng, 1996). Fu-Ling is another one of the most widely used Chinese herbal drugs. It occurs naturally as the scelerderma of Poria coccus (Schw.), a fungus which grows on the roots of pine trees. Based on its major pharmacological effects, Fu-Ling has been classified as a sedative and diuretic. In addition, Fu-Ling has been considered as one of the major ingredients for various tonic recipes.

This study focused on the possible effect of Fu-Ling on interleukin-10 (IL-10) production. IL-10 is a cytokine that shows multiple functions in a wide range of immune cells (Moore et al., 1993). mIL-10 was originally found as a cytokine product of Th2 clones. It significantly inhibits the synthesis of interferon-γ (IFN-γ) and other cytokines produced by stimulated Th1 clones (Moore et al., 1990). hIL-10, however, is produced by both Th1 and Th2 cells, and subsequently down-regulates the functions of both Th1 and Th2 cells (del Prete et al., 1993). Monocytes and macrophages are another major source of IL-10 (Wanidworanun and Strober, 1993). For monocytes and macrophages, IL-10 is an autocrine that down-regulates the expression of MHC class II, inhibits the production of proinflammatory cytokines (e.g. IL-6, TNF-α, GM-CSF, G-CSF and IL-8), suppresses NO and superoxide production and diminishes the antigen-presenting capacity (Moore et al., 1993; de Waal Malefyt et al., 1991). However, IL-10 also displays some positive effects on immune-related cells. For example, IL-10 has been demonstrated to be a differentiation factor of B-lymphocytes. Together with CD40 and other cytokines, IL-10 induces an increase in immunoglobulin production (Rousset et al., 1992). In IL-10-deficient mice, lymphocyte development and antibody response were normal, but most animals were growth retarded, anemic and suffered from chronic enterocolitis (Kuhn et al., 1993).

In this study, we observed the induction of IL-10 production by administration of Fu-Ling intraperitoneally. Using a similar protocol, we found that the immunoglobulin production by spleen cells was also regulated by Fu-Ling. Therefore, we propose that Fu-Ling might affect the function of B-lymphocytes by stimulating IL-10 production.

**Materials and Method**

*Preparation of Herbal Drug Extract*

A batch of herbal drugs was purchased from the Lao-Chen-Ge Chinese herbal drugstore, De-Hua Street, Taipei. The herbs were ground into dried powder. The Fu-Ling (5 g) powder was mixed and suspended in 100 ml of 50% ethanol. The drug suspension was boiled until half the volume of liquid remained. The suspension was then spun at 10,000 g for 30 minutes
and the supernatant was collected and dried with a Speed Vac. The dried extracts were reconstituted using phosphate buffered saline (PBS) to make a stock of 100 mg drug/ml and sterilized with a 0.2 mm Millipore filter before use.

**Animal Treatment**

For the drug treatment, the animals were divided into four groups and injected peritoneally (i.p.) with 1 ml of Fu-Ling extract, ranging from 0.1 mg, 0.5 mg to 1 mg/g body weight (mg/gw) daily for 3 days. The control group was composed of mice injected with an equal volume of PBS. Mice were sacrificed at day 4 and the spleen cells were isolated. The spleen cells (5×10^5 cells/ml) were cultured in a medium containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, antibiotics and 1 µg/ml concanavalin A (Con A) for 3 days. Culture spleen cell supernatants were collected, and the IL-10 concentrations were measured using the ELISA technique. For investigating the effects of Fu-Ling on the Ig production, the mice were treated as described. However, the spleen cells were cultured in the presence of lipopolysaccharide (LPS; 1 µg/10^5 cells) for 5 days. The immunoglobulin concentrations in the culture supernatants were estimated using the ELISA technique.

**ELISA**

For the quantitative analysis of IL-10, a cytokine ELISA set purchased from R&D Systems (MN, USA) was used. The capture antibody was a rat monoclonal antibody to mouse IL-10 and the detection antibody was a biotinylated goat anti-IL-10 polyclonal antibody. The color was developed by incubating the plate with a HRP-conjugated streptavidin (Zymed, CA, USA), followed by a substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). The reaction continued for 30 minutes at room temperature, and was stopped by adding 100 µl of 2N of sulfuric acid. The absorbence at 450 nm in each well was read using an ELISA reader (EL311, BioTek, Winooski, VT), and the data was analyzed using log-logit model.

IgA, IgG and IgM concentrations were measured using a sandwich ELISA technique. The capture antibody for the assay was a rabbit anti-mouse IgG + IgA + IgM antibody (Zymed Laboratory, CA). The secondary antibody for the assay was the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (1:500 diluted; Zymed Laboratory, CA), HRP-conjugated goat anti-mouse IgG (1:10,000 diluted; whole IgG molecule; Jackson ImmunoResearch, PA) or HRP-conjugated goat anti-mouse IgM (1:2000 diluted; heavy chain-specific; Jackson ImmunoResearch, PA). Briefly, a 96-well microtiter plate (Nunc-ImmuNo Plate, MaxiSorp, Nunc, Denmark) was precoated with 100 ng/well of capture antibodies at 4°C overnight. The plate was washed with PBS-0.05% Tween 20 solution and blocked with PBS-1% gelatin. After the blocking, the properly diluted samples and standard IgG, IgA or IgM (ranged from 1 µg/ml to 0.03125 µg/ml) were added (100 µl/well). The plate was then incubated at 37°C for 2 hours. At the end of incubation, a HRP-conjugated
secondary antibody was added (100 µl/well). After 1 hour of incubation at 37°C, the color was developed using a substrate solution containing 0.1 M citrate buffer, pH 4.5, 0.03% H₂O₂ and 0.1% of o-phenylenediamine. The absorbence at 490 nm in each well was read using an ELISA reader (EL311, BioTek, VT), and the data was analyzed using log-logit model.

Synthesis of cDNA

The level of IL-10 mRNA in the spleen cells was estimated using RT-PCR analysis 24 hours after culture without Con A. Spleen cells (1 × 10⁷ cells) isolated from Fu-Ling-treated animals were washed twice with 1 × RNase-free PBS. The cell pellet was mixed with 1 ml of TRIzol reagent (GIBCO-BRL, MD), and the mixture was forced to pass through a 25G needle five times to release RNA from the cells. This homogenate was then vigorously mixed with 0.2 ml chloroform. After sitting at room temperature for 10 minutes, the mixture was spun at 4°C for 15 minutes to separate the organic from the aqueous layers. The aqueous layers were removed into a new tube and RNA was precipitated with 0.5 ml of isopropanol. The precipitate was then resuspended in 30 µl of RNase-free water, and a 5 µl aliquot was removed for RNA quantification using GeneQuant II (Pharmacia Biotech, Piscataway, NJ). RNA in RNase-free water (2 µg in 10 µl) was mixed with 2.5 µl of oligo (dT)₁₅ solution (40 pM; Promega, WI). The solution was heated at 70°C for 10 minutes, followed by cooling at room temperature for 10 minutes and then transferred onto ice. A reaction mixture containing four dNTPs (Boehringer Mannheim, Germany), DTT (GIBCO/BRL, MD), reverse transcriptase (M-MLV; Promega, WI) and RNasin (Promega, WI) was subsequently mixed with RNA. The reaction was carried out at 37°C for 60 minutes to synthesize the cDNA.

PCR

cDNA (5 µl) was mixed with 0.5 µl 4d NTP, 10 µl primer mix (2.5 µM each), 0.5 µl Tag polymerase, 2 µl MgCl₂ (2 mM) and 5 µl PCR buffer. The DEPC-treated water was then added to make up a total volume of 50 µl. The primers (Promega, WI) used for PCR amplification were as follows: IL-10 sense primer 5’-ATGCAGGACTTTAAGGGTTACTTG-3’; IL-10 antisense primer 5’-TAGACACCTTGGCTTGGAGCTTA-3’; β₂ microglobulin (internal control) sense primer 5’-TGACCGGCTTGTATGCTATC-3’ and β₂ microglobulin antisense primer 5’-CAGTGTGAGCCAGGATATAG-3’. The PCR conditions were denaturation at 94°C for 50 seconds, annealing at 60°C for 45 seconds and primer extension at 72°C for 45 seconds. After 40 cycles (35 cycles for β₂-microglobulin) of amplification, the PCR products were subjected to gel electrophoresis through 1.5% agarose (Sigma, MO) containing ethidium bromide at 80 V. The amplicons were visualized under UV light.
Statistics Analysis

Data from the control or drug-treatment groups were tested by ANOVA. The difference between the two means was assessed using the Student’s t-test. Probability values of < 0.05 were considered to be significant.

Results

Fu-Ling Induced Increased IL-10 Production

Spleen cells isolated from the mice injected with various doses of Fu-Ling or the same volume of PBS (control group) were cultured in vitro for three days. The daily amount of IL-10 secretion by the spleen each was measured using ELISA. The results indicated that the spleen cells isolated from the mice injected with 0.5 mg and 1.0 mg/gw of Fu-Ling significantly increased levels of IL-10 secretion (Fig. 1). However, the level of IL-10 secretion induced by 1.0 mg/gw body weight was less than that induced by 0.5 mg/gw (Fig. 1).

Figure 1. Fu-Ling induced an increase in IL-10 secretion by spleen cells in vitro. BALB/c mice were peritoneally injected with various doses of Fu-Ling, ranging from 0.1 mg/gw to 1.0 mg/gw of body weight, for 3 consecutive days. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated 48 hours after treatment and then incubated for three days with 1 µg/ml of Con A. Supernatants were harvested and assayed for IL-10 by ELISA. ANOVA indicated a significant effect by Fu-Ling on the spleen cells to secrete IL-10. Data were mean ± SEM of five similar experiments.
RT-PCR was carried out to monitor the expression of IL-10 mRNA using mRNA of β2-microglobulin as the internal control. Data indicated that the IL-10 mRNA was detected in the spleen cells when the mice were injected with 0.5 mg/gw and 1.0 mg/gw (Fig. 2). However, the amount of IL-10 mRNA was not reduced in the spleen cells when the mice were injected with Fu-Ling up to 1.0 mg/gw (Fig. 2).

Figure 2. RT-PCR for the detection of Fu-Ling-induced IL-10 mRNA expression. BALB/c mice were peritoneally injected with various doses of Fu-Ling, ranging from 0.1 mg/gw to 1.0 mg/gw body weight, for 3 consecutive days. Spleen cells were isolated and in vitro cultured for 24 hours without Con A. The total mRNA was isolated from the spleen cells (1 × 10^7 cells). cDNA was synthesized and then amplified using PCR as described in the “Materials and Method” section. M: markers; Lane 1: control (PBS); Lane 2: 0.1 mg/gw; Lane 3: 1.0 mg/gw; and Lane 4: 10 mg/gw body weight. The length of the product was 254 bp for IL-10 and 222 bp for β2m.

Induction of Immunoglobulin Secretion by Fu-Ling

IL-10 has been demonstrated to be a differentiation factor of B-lymphocytes (Rousset et al., 1992). Therefore, a dose-dependent study for Fu-Ling was carried out to investigate the induction of Igs production. Under our experimental conditions, spleen cells isolated from the mice injected with 0.1 mg/gw body weight or higher doses of Fu-Ling exhibited significantly increased IgG and IgA secretions (Figs. 3 and 4). However, the effect of Fu-Ling on the IgM secretion was not significant (Fig. 5).

Discussion

Our observation in this study demonstrated that the Chinese herbal drug Fu-Ling was able to regulate antibody production by augmenting IL-10 production. Both secretion and the mRNA IL-10 level were increased in the spleen cells isolated from Fu-Ling-treated mice. IL-10 is a well-known anti-inflammatory cytokine. IL-10 down-regulates a panel of genes in monocytes including LPS-inducible genes, IFN-γ-inducible genes and IL-4-inducible
genes (Donnelly et al., 1999). Among those LPS-inducible genes, IL-10 significantly reduced TNF-α, IL-1, IL-6, IL-8, IL-12, GM-CSF and even IL-10 gene expression by itself.

Figure 3. Fu-Ling induced an increase in immunoglobulin secretion by spleen cells in vitro. BALB/c mice were peritoneally injected with various doses of Fu-Ling, ranging from 0.1 mg/gw to 1.0 mg/gw body weight, for 3 consecutive days. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 µg/ml of LPS. Supernatants were harvested and assayed for IgG concentrations at days 3, 4 and 5. Data were mean ± SEM of five similar experiments. * indicates p < 0.05 from the control group.

However, our previous studies indicated that Fu-Ling modulated cytokine production by human monocytes. Fu-Ling significantly enhanced the secretion of cytokines produced by monocytes, i.e. IL-1β, TNF-α and IL-6 but suppressed TGF-β production under in vitro conditions (Yu and Tseng, 1996). Under similar experimental conditions, our data also indicated that Fu-Ling significantly augmented GM-CSF secretions using human peripheral blood mononuclear cells (Tseng and Li, 1996).
The controversial data obtained from in vivo and in vitro trials may result from the target cells of the drug. Under in vitro conditions, the crude extract of Fu-Ling directly acts on the monocytes by either interacting with intracellular molecules or going through a receptor-mediated pathway. Under in vivo conditions, the components of Fu-Ling may not be able to directly affect the monocyte functions. Instead, Fu-Ling may act on the Th2 cells and monocytes to induce IL-10 production. The Fu-Ling-induced IL-10 subsequently reduces the production of monocyte-derived cytokines. Whether administration of Fu-Ling extract can inhibit the synthesis of monocyte-derived cytokines remains for further investigation.

In terms of immunoglobulin production, data obtained from this study were consistent with the IL-10 production. IL-10 is best known for its ability to induce B-lymphocyte differentiation (Rousset et al., 1992). An increase in IL-10 implied that more B-lymphocytes

Figure 4. Fu-Ling induced an increase in immunoglobulin secretion by spleen cells in vitro. BALB/c mice were peritoneally injected with various doses of Fu-Ling, ranging from 0.1 mg/gw to 1.0 mg/gw body weight, for 3 consecutive days. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 μg/ml of LPS. Supernatants were harvested and assayed for IgA concentrations using ELISA at days 3, 4 and 5. Data were mean ± SEM of five similar experiments. * indicates p < 0.05 from the control group.
were differentiated from the IgM producer to the IgG or IgA producers. Our data supported the hypothesis that spleen cells isolated from Fu-Ling-treated mice did show an increase in both IgG and IgA secretions but no increase in IgM.

![Graph A](image1)

**Figure 5.** Fu-Ling induced an increase in immunoglobulin secretion by spleen cells *in vitro*. BALB/c mice were peritoneally injected with various doses of Fu-Ling, ranging from 0.1 mg/g to 1.0 mg/g body weight, for 3 consecutive days. The control group consisted of mice injected with an equal volume of PBS. Spleen cells were isolated after the treatment and then incubated for 5 days with 1 mg/ml of LPS. Supernatants were harvested and assayed for IgM concentrations using ELISA at days 3, 4 and 5. Data were mean ± SEM of five similar experiments. * indicates p < 0.05 from the control group.

![Graph B](image2)

This observation was also inconsistent with the data obtained from the *in vitro* study on the human blood cells (Lu *et al.*, 1994). Under *in vitro* conditions, Fu-Ling significantly induced an increase in IgM secretions using human peripheral blood mononuclear cells but had less effect on IgA and IgM secretions. This might result from the failure of Fu-Ling
alone to induce B-cells in human blood to undergo differentiation, and most of the B-cells in peripheral blood were resting cells.

IL-10 is one of the potent inhibitors of inflammatory response. Therefore, our observation may provide a convincing interpretation of why Fu-Ling acts as a powerful drug to reduce edema and to enhance the immune response. Our previous report (Yu and Tseng, 1996) and current data suggested that a steroid-type compound isolated from Fu-Ling extract may be the biologically active ingredient that regulates cytokine production. It will therefore be possible to isolate a therapeutic compound from Fu-Ling extract.

References


