Astragalus mongholicus and Polygonum multiflorum’s Protective Function Against Cyclophosphamide Inhibitory Effect on Thymus

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Abstract: The protective effects of two Chinese herbs, Astragalus mongholicus, Polygonum multiflorum and Astragalus mongholicus-Polygonum multiflorum in combination against thymus injury induced by cyclophosphamide were evaluated by transmission electron microscopy, image analysis, DNA gel electrophoresis as well as flow cytometry. Results showed that mice pretreated with cyclophosphamide had degenerated thymus with less normal thymocytes; when those mice were treated with the herbs, thymus morphology improved. The apoptosis analysis showed the thymus treated with the herbs had fewer apoptotic thymocytes than the thymus pretreated with cyclophosphamide only. In conclusion, Astragalus mongholicus and Polygonum multiflorum have protective effects on the thymus against cyclophosphamide-induced injury. Their protective effects partly attribute to reduced apoptosis. Astragalus mongholicus-Polygonum multiflorum in combination has better effects than either of the two herbs.

Keywords: Astragalus mongholicus; Polygonum multiflorum; Cyclophosphamide; Thymus; Apoptosis.

Introduction

Chemotherapy is often given to patients as a standard treatment for some types of cancer and infectious disease. Serious side effects such as a decrease in blood cell counts (white blood cells, red blood cells and platelets) may occur after treatment, and often lead to the...
discontinuity of chemotherapy. Therefore, for the success of chemotherapy, it is important to prevent and antagonize those side effects. Cyclophosphamide is one of the most popular agents in chemotherapy. It is used to treat a type of leukaemia known as chronic lymphocytic leukemia, lymphomas, ovarian cancer and bladder cancer (Wexler et al., 2000). It is also generally used to construct an immunodepressive animal model (Casteels et al., 1998; Li et al., 2000).

*Astragalus mongholicus* and *Polygonum multiflorum* are Chinese herbs commonly used in the clinic. They can improve both cell immunity and humoral immunity. *Astragalus mongholicus* can improve the IgM and IgE, stimulate the lymphocyte to produce interferon, enhance the natural killer cell (NK) activity, and improve the Fc receptor expression of macrophages. *Polygonum multiflorum* can improve T, B lymphocyte activity and enhance the macrocytosis of macrophage in abdominal cavity (Lin, 2001).

Some reports showed that *Astragalus mongholicus* could prevent and control the cyclophosphamide-induced impairment such as low white cell count in peripheral blood and nucleated cell decrease in bone marrow (Augstein et al., 1998; Wei et al., 1993; Wu et al., 1999, Zhang et al., 1999).

*Astragalus mongholicus* injection and *Polygonum multiflorum* powder are two commercially available drugs for fatigue patients. It is unknown weather they have their effects on cancer patients. In this report, these two drugs were used on doses of 6 g/kg/d, equivalent to human clinical dose, to study their effects on cyclophosphamide-treated mice. The main focus was on the thymus improvement of *Astragalus mongholicus* and *Polygonum multiflorum*. We attempted to find evidence to support their use in cancer treatment.

**Materials and Methods**

**Main Reagents**

*Astragalus mongholicus* injection (1 g/ml) was purchased from Liuhe Pharmaceutical Company, Jiangsu, China; *Polygonum multiflorum* powder (Chinese Medicine Institute) diluted to 0.5 g/ml in double distilled water (0.1 g/ml). Cyclophosphamide was purchased from the 12th Pharmaceutical Company of Shanghai, Shanghai, PRC.

**Animal and Treatment**

Kun Ming mice (50, each weighting 25–30 g), provided by the Laboratory Animal Center of the Shantou University Medical College, were gender controlled and divided evenly into five groups. During 14-day experimental periods, the mice were treated with cyclophosphamide (8 mg/kg, i.p.), every other day, to construct the immune impairment. At the same time, some mice were treated with *Astragalus mongholicus* (6 g/kg/day, gavage) — designated as A group, *Polygonum multiflorum* (6 g/kg/day, gavage) — designated as P group, *Astragalus mongholicus*/*Polygonum multiflorum* (6 g/kg/day, gavage) — designated as D group, and vehicle (no treatment) — designated as C group, respectively. Another normal control group without any treatment was also set — designated as O group.
Macroscopic Evaluation

Mice were sacrificed by cervical dislocation at least 24 hours after the last treatment. Thymus was collected and weighed to count thymus indices.

HE Staining

Thymus from different groups was routinely prepared for histological evaluation. Routine hematoxylin & eosin (HE) staining of paraffin embedded slices was used to make lymphocytes visible.

Immunohistochemical Staining

Primary antibody lysozyme (DAKO Company) was used to stain the macrophages in thymus. Primary antibody keratin (DAKO Company) was used to stain epithelial reticular cells in thymus. Both applied the staining procedure of avidin-biotin complex (ABC) according to the manufacturer’s instructions.

Image Analysis

Images from both HE sections and immunohistochemical sections were captured by Sony 3CCD Color Video Camera and processed by image pro plus 4.5 image analysis software. The following parameters were acquired and analyzed: the count and area of lymphocytes in HE sections, the count, area and gray scale of macrophages in lysozyme staining sections and the keratin-positive cell perimeter, area and gray scale in keratin staining sections.

Flow Cytometry

Fresh thymus was minced and filtrated through 100 sieving mesh to collect the single thymus cell. Single thymus cell suspension was centrifuged (200 g) and the cell droplet was fixed by 70% cold ethanol for 24 hours at 4°C, digested by RNase (50 mg/l) for 1 hour at 37°C. Propidium iodide (PI, 50 mg/l) stained for 1 hour at 4°C. Cell suspension was detected by FACSort (Becton Dickinson Company) for the cellular DNA content.

DNA Gel Electrophoresis

The dissociated single thymus cell was washed in phosphate-buffered saline (PBS, pH 7.4) and resuspended in 1 ml of lysis buffer (50 mM Tris pH = 8.0, 10 mM NaCl, 100 mM EDTA pH = 8, 1% SDS, 100 µg proteinase K/ml) at 37°C for 1 hour. Lysate was deproteined with distilled phenol and water phase separated. Then, equal volume solution of chloroform/isoamylalcohol (= 24/1) was added, carefully mixed, spun at 12,000 g/5 minutes/4°C and the water phase with DNA was separated. Optical density (OD) was measured for each
specimen to determine the concentration of DNA; 5 µg DNA specimen mixed with carrier buffer (5% bromophenol blue contained) and 8 µg DNA maker (PGEM-32f (+)-Hae III) were applied on an agarose gel. Neutral gel electrophoresis (1.8% agarose, 3 V/cm) was proceeded in TBE buffer (5.4 g Tris base, 2.75 g boric acid, 0.5 M EDTA pH = 8 in 1000 ml H2O). Then the gel was stained with ethidium bromide (1 µg/ml) water bath.

Transmission Electron Microscopy

Single thymus cell suspension was centrifuged (200 g) and the cell droplet was fixed by glutaraldehyde (25 g/l) and osmium tetroxide (20 g/l), dehydrated by gradient ethanol, embedded in Epon 812 (epoxy resin 812) and sectioned, then stained with uranyl acetate and lead citrate. It was observed under a Hitachi H600 transmission electron microscope.

Statistical Analysis

All data were statistically analyzed by the T test method. P < 0.05 was considered statistically significant.

Results

Macroscopic Pathology

Thymus weight and thymus index (thymus weight in mg/100 g body weight) in the C group (17.25 ± 5.64, 72.54 ± 30.57) were significantly lower than in the O group (32.13 ± 5.06, 133.21 ± 23.76) (p < 0.01). Thymus weight and thymus index in the A group (32.34 ± 5.24, 133.09 ± 25.14), P group (31.61 ± 7.10, 128.13 ± 28.40) and D group (37.75 ± 11.27, 157.55 ± 32.71) were significantly higher than that in the C group (p < 0.01). There is no significant difference between the A, P, D and O groups (p > 0.05).

Microscopic Pathology

The HE staining showed no distinctive morphological changes among O, C, A, P and D groups. In the C group, the cell counts of lymphocyte were lower compared to other groups. Macrophages were round, spindle-like and irregular, with brown lysozyme-positive particles in cytoplasm. Lysozyme staining also showed no distinctive morphological changes among groups, except fewer and lighter staining macrophages appeared in the C group (Fig. 1A). Keratin-positive cells formed web-like structure. Destruction of web-like structure was more popular in the C group than in the other groups. Brown keratin-positive particles could be seen in the cytoplasm and dendrites of epithelial reticular cells. Keratin staining showed weaker staining in the C group than in other groups (Fig. 1B).

Cell counts and area of thymus lymphocytes were significantly lower and smaller in the C group than in the O group (p < 0.01). It was also significantly higher and larger in the A,
P and D groups when compared to the C group (p < 0.01). In contrast, there was no significant difference among the A, P, D and O groups (p > 0.05) (Fig. 2). Cell counts and area of thymic medullar macrophages were significantly higher and larger in the O, A, P and D groups than in the C group (p < 0.01); conversely, the gray scale was significantly lower in the O, A, P and D groups than in the C group (p < 0.01). There was no significant difference among the A, P, D and O groups (p > 0.05) (Fig. 3). Thymic medullar epithelial reticular cell perimeter and area were also higher and larger in the O, A, P and D groups than in the C group (p < 0.01). Gray scale was lower in the O, A, P and D groups than in the C group (p < 0.01). There was no significant difference in gray scale among the A, P, D and O groups (p > 0.05). The perimeter of the A and P groups were lower than that in the O group (p < 0.05, p < 0.01), and no significant difference was found between the D and O groups (p > 0.05). The cell area was remarkably smaller in the A and P groups than in the O group (p < 0.01), and was smaller in the D than in the O groups (p < 0.05) (Fig. 4).

Ultrastructure

In the O group, thymocytes were round or oval with eccentrically positioned heterochromatin. Nucleoli were distinctive (Fig. 5a) and metaphase of cell division was also popular. In the C,
Figure 2a and b. Image analysis of thymus lymphocytes.
Figure 3a to c. Image analysis of thymic medullar lysozyme-positive macrophages.
Figure 4a to c. Image analysis of thymic medullar keratin-positive epithelial reticular cells.
A, P and D groups, different phases of apoptotic cells could be seen (Fig. 5b). Earlier apoptotic cells had distinctive translocation of nucleus and condensed chromatin to crescent shape and arc shape just beneath the nuclear membrane in the D group (Fig. 5c). Apoptotic changes could be seen in the A and P groups such as slighter pyknosis and earlier karyorrhexic fragments of nucleus into two pieces of membrane bound or free particles (Figs. 5d and e). Apoptotic cells in the C group showed a variety of morphological changes such as later pyknosis and karyorrhexis, and even autosome formation (Fig. 5f).

**Flow Cytometry for Cellular DNA Contents**

DNA-FCM with PI revealed a sub-population of cells, designated apoptotic cells, with reduced DNA stainability, in groups of C, A, P and D. The peak is below the normal G0/G1 region. It is believed that the reduced DNA stainability is the consequence of progressive loss of DNA from apoptotic cells. The ratio of apoptotic cells, represented by area under apoptotic cells curve/area under G0/G1 curve were ranked: D group (5.12 ± 0.36) < A group (20.44 ± 1.29) < P group (24.27 ± 0.16) < C group (43.47 ± 2.03). It was significant when the ratio of apoptotic cells in the A, P and D groups were compared to the C group (p < 0.01), and when the A and P groups were compared to the D group (p < 0.01). No apoptotic cells were revealed in the O group.
Typical nucleosomal ladder of total genome DNA gel electrophoresis appeared in the C, A, P and D groups (Fig. 6).

**Discussion**

The reduced DNA stainability of apoptotic cells distinguishes them from necrotic or vital cells. In the case of apoptosis, DNA–FCM shows a distinct cell subpopulation below the G0/G1 region of normal diploid cells. This phenomenon is often applied in apoptosis analysis (Vermes et al., 2000; Ormerod et al., 1993).

During apoptotic cell death, cellular endonucleases cleave genomic DNA between nucleosomes to produce fragments whose lengths vary by multiples of 180–200 bp. When resolved using agarose gel electrophoresis, these DNA fragments appear as a nucleosomal ladder, a widely recognized hallmark of apoptosis (Matalova and Spanova, 2002). The cell ultrastructure is also available for further verification of apoptosis (Kerr et al., 1994; Sun et al., 2001). Thus, the methods for apoptosis analysis in this report are reliable.

Cyclophosphamide is generally applied as an immune inhibitory medicine with remarkable cytotoxicity (Zhang et al., 1999; Cai et al., 1997; Casteels et al., 1998; Pette et al., 1995). Some reports demonstrated that cyclophosphamide increased the apoptotic cells in thymus and some tumor cells (Wexler et al., 2000). In this report, the cyclophosphamide-induced apoptosis was verified by means of flow cytometry of cellular DNA contents, thymus genome DNA gel electrophoresis and thymus ultrastructure. After treatment with *Astragalus mongholicus* and/or *Polygonum multiflorum*, mice had better thymus weight, thymus index and thymus thymocytes count and area. DNA-FCM results also showed *Astragalus mongholicus* and/or *Polygonum multiflorum* lowered cyclophosphamide-mediated cell apoptosis in thymus. Therefore, *Astragalus mongholicus* and *Polygonum multiflorum* can
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protect thymus against cyclophosphamide-mediated immune impairment. This was consistent with previous reports (Wei et al., 1993; Wu et al., 1999; Lin, 2001).

The balance between the proliferation and cell death is very important to maintain the tissue and organ in normal condition. Too much apoptosis can result in immune deficiency (Amesen, 1992). In this report, the data showed that the thymocytes apoptosis induced by cyclophosphamide was retarded after treatment with *Astragalus mongholicus* and/or *Polygonum multiflorum*.

Retardation in apoptotic thymocytes might be related to the improved thymic epithelial cell macrophage and other nurse cells in the thymus. Some reports demonstrated thymic nurse cells served as the site of thymocyte development in the thymus (Hiramine et al., 1996). In a previous report, we also observed that *Astragalus mongholicus* and *Polygonum multiflorum* could reverse the morphological change of aged mice (Wei et al., 1991 and 1993). So here, we hypothesized that *Astragalus mongholicus* and *Polygonum multiflorum* could improve the thymocyte development and inhibit thymocytes apoptosis as well.

In this report, *Astragalus mongholicus-Polygonum multiflorum*, in combination, showed better effect than either *Astragalus mongholicus* or *Polygonum multiflorum* alone, which demonstrated that *Astragalus mongholicus* and *Polygonum multiflorum* have synergetic or additive effects against thymus injury.

References


