A Chinese herbal decoction, Danggui Buxue Tang, activates extracellular signal-regulated kinase in cultured T-lymphocytes

Qiu T. Gao\textsuperscript{a}, Jerry K.H. Cheung\textsuperscript{a}, Jun Li\textsuperscript{a}, Zhi Y. Jiang\textsuperscript{a}, Glainice K.Y. Chu\textsuperscript{a}, Ran Duan\textsuperscript{a}, Anna W.H. Cheung\textsuperscript{a}, Kui J. Zhao\textsuperscript{a,b}, Roy C.Y. Choi\textsuperscript{a}, Tina T.X. Dong\textsuperscript{a}, Karl W.K. Tsim\textsuperscript{a,*}

\textsuperscript{a} Department of Biology and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong
\textsuperscript{b} Beijing Friendship Hospital, Affiliate of Capital University of Medical Sciences, 95 Yong An Road, Beijing 100050, China

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\begin{abstract}
Danggui Buxue Tang (DBT) is prepared from Radix Astragali and Radix Angelicae Sinensis. This Chinese herbal decoction has been shown to stimulate the proliferation of T-lymphocytes; however, the action mechanism of this stimulation has not been revealed. In cultured T-lymphocytes, application of DBT markedly induced the cell proliferation, the release of interleukin-2, -6 and -10, as well as the phosphorylation of extracellular signal-regulated kinases (ERK). The pre-treatment of ERK inhibitor blocked the DBT-induced immune responses. In addition, the polysaccharide-enriched fraction of DBT showed marked responses on the cultured T-lymphocytes suggesting the important role of DBT polysaccharide in triggering such immune responses.
\end{abstract}

\begin{keywords}
Danggui Buxue Tang; Radix Astragali; Radix Angelicae Sinensis; T-lymphocyte; Cytokine; ERK
\end{keywords}

1. Introduction

Among thousands of herbal formulae from traditional Chinese medicine (TCM), Danggui Buxue Tang (DBT; a herbal decoction) is a simple combination of only two herbs. DBT was first described in \textit{Neiwaishang Bianhuan Lun} by Li Dongyuan in China in AD 1247. \textit{Li} described the DBT formula should include: 10 qian of Radix Astragali (RA), roots of \textit{Astragalus membranaceus} (Fisch.) Bunge or \textit{Astragalus membranaceus} (Fisch.) Bunge var. \textit{mongholicus} (Bunge) P.K. Hsiao, and two qian of Radix Angelicae Sinensis (RAS), roots of \textit{Angelica sinensis} (Oliv.) Diels. One qian equals about 3 g. The mixed herbs were boiled in two bowls (~500 ml) of water over moderate heat until the final volume was reduced by half. Traditionally, DBT has been prescribed to women in China as a remedy for menopausal symptoms. These women are directed to drink DBT daily to raise their \textit{Qi} (vital energy) and nourish their \textit{Blood} (body circulation). RA has been proven to be an immunostimulant, hepatoprotective, anti-diabetic, analgesic, expectorant and sedative drug [1]. On the other hand, RAS is used to invigorate the blood circulation in treating menstrual disorders and to modulate the immune system [2,3]. Although chemical and biological analyses have been performed on the individual herbs [4,5], the rationale for having two herbs in DBT has never been explained; this consequently hinders the development of multi-herb decoctions as disease and disorder remedies.

By determining the chemical and biological properties of DBT, the optimized conditions for extraction have been established [4,6], which, interestingly, are in accord with the weight ratio of 5:1 for RA to RAS in the ancient preparation. In addition, a standardized DBT was established by revealing the amounts of RA-derived calycosin and formononetin, and RAS-derived ferulic acid and ligustilide in the decoction [5,7].

The immuno-regulatory property (one of the \textit{Qi} functions) of DBT was determined here. Previous studies indicated that the proliferation of T-lymphocytes could be stimulated by the treatment of DBT; however, the molecular event responsible for this stimulation has not been determined. To reveal the action mechanism of this ancient herbal decoction, we treated cultured T-lymphocytes with DBT and, subsequently, analyzed the releases of ten cytokines including interleukin (IL)-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, granulocyte–macrophage colony stimulating factor (GM-CSF), interferon-\textgreek{g} (IFN-\textgreek{g}), tumor necrotic factor-\textgreek{x} (TNF-\textgreek{x}) and the phosphorylation of extracellular signal-regulated kinases (ERK)1/2. In addition, the role of DBT polysaccharide and its signaling pathway for the DBT-induced cytokine release was elucidated.

2. Materials and methods

2.1. Preparation and standardization of DBT

Three-year-old \textit{A. membranaceus} var. \textit{mongholicus} roots from Shaxi Province [8] and two-year-old \textit{A. sinensis} roots from Minxian in Gansu Province [3] were collected in 2002. In preparing DBT, exact amounts of RAS and RA were weighed according to a ratio of 5:1 and then mixed well in a vortex. The mixture was boiled in 8 vol of water (\textit{v/v}) for 2 h and extracted twice; this extraction followed the ancient recipe that had been shown to have the best extracting condition [4,6]. Separate samples of RAS and RA were extracted by the same method. DBT polysaccharides were separated into polysaccharide-enriched fraction and polysaccharide-depleted fraction by precipitating the DBT extract with 70\% ethanol. The two fractions were separated after centrifugation at 4000 \texttimes g for 20 min. The polysaccharide-enriched fraction accounts for ~18\% of the total dried weight. All the fractions were dried by lyophilization and stored at \textdegree 80 \textdegree C.

Abbreviations: TCM, Traditional Chinese medicine; DBT, Danggui Buxue Tang; RA, Radix Astragali; RAS, Radix Angelicae Sinensis; ERK, extracellular signal-regulated kinase
Standardization of DBT was done by HPLC profiling and the contents of ferulic acid, calcyosin, formononetin and ligustilide (z-isoflavan). AR- and HPLC-grade reagents were from Merck (Darmstadt, Germany). A Waters (Milford, MA) HPLC system consisting of a 600 pump, a 717 auto-sampler and a UV/VIS Photodiode Array 2998 detector was used for all analyses. The chromatographic separations were carried out on a DELTA-PAK C18 (particle size 4.6 μm, 3.9 mm × 150 mm) with acetonitrile (as Solvent A) and 0.01% phosphoric acid (as Solvent B) in the mobile phase at a flow rate of 1.0 ml/min at room temperature, as described previously [5–7].

2. Results

3.1. Standardization of DBT

By discovering the amounts of two chemical markers in RA (calycosin and formononetin) and two others in RAS (ferulic acid and ligustilide), we were able to standardize the optimal DBT. The standardized DBT should contain 0.186 mg calycosin, 0.155 mg formononetin, 0.351 mg ferulic acid and 0.204 mg ligustilide per 1 g dried weight of DBT, which was in line with our previous calibration [5,7]. From the extraction efficiency calculations, we found that the yield of DBT in this weight ratio was 32 ± 3% (n = 5). These parameters established the chemical standards and the optimal DBT mixture for the remaining biochemical experiments.

3.2. DBT induces T-lymphocyte proliferation

In order to reveal the possible immune-regulatory functions of DBT, water extracts of DBT, RA, RAS, and RA + RAS (boiled separately and then mixed together in 5:1 ratio) were applied onto the cultured human T-lymphocytes, and the cell proliferation was determined. All groups showed significant stimulatory effect on T-lymphocyte proliferation (Fig. 1). The effects of different herbal extracts showed a good dose-dependent response. At the dosage of 0.1 mg/ml, DBT showed the sub-maximal effect, which was significantly higher than the other groups. Higher amount of the extracts did not show significant increase in the cell proliferation, suggesting a saturation effect. The addition of RA + RAS (boiled separately) showed a much lower activation effect on the T-lymphocyte proliferation.

Fig. 1. Effects of extracts from RA, RAS and DBT in cultured T-lymphocytes. DBT, RA, or RAS, or RA + RAS at different amounts were applied onto cultured T-lymphocytes for 3 days. PHA (10 μg/ml) was used as a positive control. Values of cell proliferation (by XTT assay) are expressed in percentage of increase as compared to control cultures (without herbal extract). Values are in means ± S.E.M., where n = 5, each with triplicate samples.

2.4. Determination of ERK1/2 phosphorylation

Cultured T-lymphocytes were treated with DBT for 0, 10, 20, 30, 45, 60 min, respectively. To investigate the effect of inhibitor, the cultures were serum starved with or without the inhibitor for 3 h before the drug applications. After the drug treatments, the cultures were collected immediately in lysis buffer (125 mM Tris–HCl, 2% SDS, 10% glycerol, 200 mM 2-mercaptoethanol, pH 6.8), and the proteins were subjected to SDS-PAGE analysis. Phosphorylated ERK1/2 were recognized by anti-phospho-ERK1/2 (Santa Cruz, CA, USA) and anti-total ERK1/2 antibody (1:5000; Cell Signaling, Danvers, MA) at 4 °C for 12 h, and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5000; Invitrogen) for 1 h at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence (ECL) method (GE Healthcare; Piscataway, NJ). The band intensities, recognized by the antibodies in the ECL film, in control and agonist-stimulated samples were run on the same gel and under strictly standardized ECL conditions. The bands were compared on an image analyzer, using in each case a calibration plot constructed from a parallel gel with serial dilution of one of those samples: this was to ensure the sub-saturation of the gel exposure.

2.5. Other assays

The protein concentrations were measured routinely by Bradford’s method with a kit from Bio-Rad Laboratories (Hercules, CA). Statistical tests were made by the PRIMER program, version 1 (Primer of Biostatistics): differences from basal or control values (as shown in the plots) was deemed as significant (*) where P < 0.05, (**) where P < 0.01 and highly significant (****) where P < 0.001 by Student’s t-test.
3.3. DBT induces cytokine production in T-lymphocytes

Cultured T-lymphocytes were treated for 3 days with extracts derived from DBT, RA, RAS, or RA + RAS. The release of cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF, IFN-γ and TNF-α) from the drug-treated T-lymphocytes was determined by cytokine antibody array. By comparing to the control cultures, the releases of IL-2, IL-6, IL-8 and IL-10 were markedly induced by DBT (Fig. 2A). Amongst the four cytokines, IL-2, IL-6 and IL-10 showed the specificity for DBT, i.e. not induced by RA, RAS or RA + RAS, and thus they were selected for further analysis. The treatment of DBT caused an increase in the release of IL-2 to ~3-fold, IL-6 to ~120-fold and IL-10 to ~20-fold. Interestingly, the treatment of RA, RAS or a simple mixture of RA and RAS did not change the release of the cytokines suggested that boiling of two herbs together is essential. The levels of IL-4, IL-5, IL-13, GM-CSF, IFN-γ and TNF-α were not affected by the treatment of DBT, RA, RAS, and RA + RAS.

The releases of IL-2, IL-6 and IL-10 were quantified by an ELISA assay. Fig. 2B and C shows a dose–response curve for the releases of cytokines after DBT treatment. The responses of the three cytokines were rather similar, i.e. 0.1 mg/ml of DBT in all cases induced the release to a maximum; the maximal induction for IL-2 was at ~4-fold, IL-6 at ~20-fold, and IL-10 at ~8-fold. By comparing the results from the cytokine array analysis, the results from ELISA assay revealed a lower value, which could be accounted by the sensitivity of the two different assay systems.

3.4. DBT induces ERK phosphorylation

Activation of ERK, a member of mitogen-activated protein (MAP) kinases that acts as a key signal for T-lymphocyte proliferation, was determined in DBT-treated cells. By using antibodies specific for phosphorylated ERK and total ERK, the phosphorylations of the ERK1 (~44 kDa) and ERK2 (~42 kDa) in cultured T-lymphocytes were revealed: both of the phosphorylations were increased by application of DBT (Fig. 3A and B). The maximal induction of ~10-fold on ERK phosphorylation was revealed after 30 min of DBT application. The ERK activation was transient, which fully returned to a low level after 60 min of the treatment. The application of TPA, served as a control, significantly induced ERK1/2 phosphorylation at a more sustained manner, which is more robust than that of DBT. The DBT-induced ERK phosphorylation at 30 min was fully blocked by pre-treating the cultures with U0126 (20 μM; a MEK inhibitor) (Fig. 3C and D).

![Fig. 2](image-url)
To further investigate whether the immune-regulatory effects of DBT were related to the ERK1/2 phosphorylation, the release of cytokines in the DBT-treated T-lymphocytes in the presence of U0126 was tested. The DBT-induced T-lymphocyte proliferation was fully blocked by U0126 (Fig. 4). In parallel, the releases of IL-2, IL-6 and IL-10, induced by DBT, were blocked by the pre-treatment of U0126 (Fig. 4). These results strongly showed that the pre-treatment of MEK inhibitor could fully abolish the immune-regulatory effects of DBT on T-lymphocytes, and which suggested the crucial role of ERK in mediating the immune responses of DBT.

3.5. The polysaccharide of DBT is an immune activator

In order to identify the active component within DBT in triggering the aforementioned immune activations, two fractions: polysaccharide-enriched (~18% of the dried weight) and polysaccharide-depleted (~82% of the dried weight) fractions were obtained. The polysaccharide-enriched fraction, when applied onto the cultured T-lymphocytes, showed a marked increase in its immune activation effect. At 50 μg/ml

Fig. 3. DBT induces ERK phosphorylation in T-lymphocytes. (A) Culture T-lymphocytes were serum starved for 3 h before the addition of DBT (0.1 mg/ml) for different time. Total and phosphorylated ERK1/2 were revealed by using specific antibodies. TPA (0.1 μM) was used as a positive control. (B) The quantitation of phosphorylation from the blots in (A) by calibrating the densitometry. (C) The phosphorylation assay was performed on the cultured T-lymphocytes as in (A), except the cultured was pre-treated with U0126 (20 μM) for 3 h before the application of DBT for 30 min. DMSO (a solvent for U0126 at 0.1%) served as a control. (D) The quantitation of phosphorylation from the blots in (C) by calibrating the densitometry. Values are expressed as the ratio to basal reading where time 0 (untreated as basal) equals to 1, and in means ± S.E.M., where n = 4.

Fig. 4. The DBT-induced immuno-modulating effects are mediated by ERK1/2. T-lymphocytes were pre-treated with buffer (0.1% DMSO), or 20 μM U0126, for 3 h before the addition of DBT (0.1 mg/ml) for 3 days. The proliferation of T-lymphocyte was assayed by XTT, and the amounts of cytokines were determined by ELISA assay as in Fig. 3. Values are expressed as the ratio to basal reading where the untreated culture equals to 1, and in means ± S.E.M., where n = 4. **P < 0.01 and ***P < 0.001 as compared to the buffer control.
of polysaccharide-enriched fraction, the stimulation of cell proliferation was increased by over 50% that was 5-fold higher than that of polysaccharide-depleted fraction (Fig. 5A). In parallel, the polysaccharides-enriched fraction showed a higher activation effect in stimulating the release of cytokines. At 50 μg/ml of polysaccharide-enriched fraction, the releases of IL-2, IL-6 and IL-10 were increased by 4-fold, 20-fold and 6-fold, respectively (Fig. 5B–D). In comparison, the polysaccharides-depleted fraction could only slightly induce the cytokine release.

The phosphorylations of the ERK1 (44 kDa) and ERK2 (42 kDa) in cultured T-lymphocytes were increased by application of the polysaccharides-enriched fraction (Fig. 6A and B). Similar to the effect of DBT, the maximal induction of ~12-fold on ERK phosphorylation was revealed after 30 min of the polysaccharides-enriched fraction of DBT application. The effects of DBT polysaccharides were also fully blocked by pre-treating the cultures with U0126 (Fig. 6C and D). In contrast, the polysaccharides-depleted fraction prepared from DBT did not show activation in the ERK phosphorylation (Fig. 6A and B). The signaling pathway of DBT is similar to that of its polysaccharide, and which indicates the role of polysaccharide in triggering the immune responses.

4. Discussion

DBT, an ancient Chinese herbal decoction having a combination of RA and RAS in a weight ratio of 5:1, possesses much better effect in stimulating cultured T-lymphocytes as compared to that of the extracts derived from RA, or RAS, or RA + RAS (boiled separately and then mixed together in 5:1 ratio). In parallel to this effect, cytokine array analysis revealed a specific set of cytokines including IL-2, IL-6 and IL-10 being stimulated to release from the cultured T-lymphocytes by DBT; the cytokine release was not triggered by RA alone, or RAS alone, or RA + RAS. In addition, the insufficient stimulating effect of RA + RAS in cultured T-lymphocytes suggests that boiling of the two herbs together is essential; this method of DBT preparation, indeed, has long been recommended by TCM practitioners. Besides the effect in T-lymphocytes, the role of the correct weight ratio of RA to RAS in making the formulation of DBT has also been demonstrated in cultured breast cancer [7], macrophage [5] and osteoblast [4].

Why does DBT need the boiling of two herbs together and at a ratio of 5:1? The author of DBT, Li Dongyuan, one of the four well-known TCM practitioners in Jin and Yuan Dynasty (the period from 1115 to 1368 A.D.), wrote down the formulation, probably based on accumulated experience in clinical application. The theoretical framework of Chinese medicine is the five elements: Metal, Wood, Water, Fire and Earth. The five elements represent different organs of our body and work harmoniously to keep the body balance [10]. Li was a great believer of “School of Reinforce Earth” that is corresponding to the organ of Spleen and the function of Qi; both of them are referring to the immune functions. According to Yijing, a book of ancient Chinese philosophy, Earth represents...
the number of five. Thus, Li believed that when RA and RAS were combined at a ratio of 5:1, DBT should have the best effect in reinforcing Qi and nourishing Blood for the menopausal symptoms.

Two possible hypotheses could explain the unique biological functions of DBT. First, DBT might contain new chemicals or larger amount of active constituents than those in the extracts of RA or RAS alone. The boiling of RA and RAS together might enhance the solubility of new chemicals, and the new chemicals are only soluble in DBT. These additional chemicals could be responsible for the DBT-specific effects. On the other hand, the optimized ratio of the two herbs in yielding more active ingredients can be another good explanation for the DBT-specific effects. The present results suggested that the polysaccharide fraction should contain one of the active ingredients of DBT. In line with that, chemical analyses showed that the content of polysaccharides in DBT was much higher than just adding RA and RAS together [4]. In addition, higher amounts of RA-derived calycosin, formononetin and RAS-derived ferulic acid were also found in DBT decoction [4]. Second, there could be a synergistic effect of different components in DBT; this synergistic effect is not present in the extracts of the single herbs. This hypothesis is being supported by the fact that the polysaccharide-enriched fraction could not fully account the activity of DBT, in particular the enrichment of the activity within the polysaccharide-enriched fraction could not be revealed here. Currently, we still do not have a direct evidence to explain why DBT required the two different herbs.

DBT possesses significant effect on T-lymphocyte proliferation, and this effect, possibility, could be a result of the increased releases of IL-2, IL-6 and IL-10. This notion is strongly supported by the effect of MEK inhibitor that blocked the cytokine release as well as the proliferation of T-lymphocyte. Cytokines are a set of soluble proteins that play a key role in immuno-regulation in both innate and adaptive immune responses [11,12]. Among the DBT-specific activated cytokines, IL-2 is an important growth factor for T-lymphocyte, which could amplify lymphocyte responses in vivo [13]. The up regulation of IL-2 by DBT might be one of the important mechanisms for DBT’s effect on T-lymphocyte proliferation. IL-10 is an important growth factor for B lymphocytes and is responsible for their differentiation; it can induce immunoglobulin secretion by B lymphocyte [14,15]. Besides, IL-10 is a potent stimulator of NK cells, a function involved in inflammation that might contribute to the clearance of the pathogen and facilitate antigen acquisition from dead cells [16]. IL-6 is a multifunctional cyto-

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**Fig. 6.** DBT polysaccharide induces ERK phosphorylation in T-lymphocytes. The T-lymphocyte cultures were treated with polysaccharide-enriched (PS-enriched at 50 µg/ml) and polysaccharide-depleted (PS-depleted at 50 µg/ml) fractions for 3 days as in Fig. 4. (A) Total and phosphorylated ERK1/2 were revealed by using specific antibodies. TPA (0.1 µM) was used as a positive control. (B) The quantitation of phosphorylation from the blots in (A) by calibrating the densitometry. (C) The phosphorylation assay was performed on the cultured T-lymphocytes as in (A), except the cultured were pre-treated with U0126 (20 µM) for 3 h before the application of the fraction for 30 min. DMSO (a solvent for U0126 at 0.1%) served as a control. (D) The quantitation of phosphorylation from the blots in (C) by calibrating the densitometry. Values are expressed as the ratio to basal reading where time 0 (untreated as basal) equals to 1, and in means ± S.E.M., where n = 4.
kine that plays important roles not only in the immune system, but also in hematopoiesis, and acute phase reactions [17]. In addition to the aforementioned in vitro properties of DBT, the effects of DBT has also been tested and proven in previous animal studies. In DBT-administered mice, the formulation with RA and RAS at 5:1 ratio was the most effective decoction in triggering the immune responses; these responses included the amount of lymphocytes, macrophages and the release of IL-2 [18,19].

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