Studies on the Cytokine Production Regulation in Human Astrocytes by Yuldahansotang

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초 록

人間腦星狀細胞에서 熱多寒少湯에 의한
細胞活性物質 生成 調節에 關한 研究

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사상의학적 견지에서 太陰人의 중풍, 치매와 같은 신경계질환에 다용되고 있는 熱多寒少湯은 최근에 그 임상적 효과를 뒷받침할 다각적인 연구들이 이루어지고 있음에도 불구하고 그 정확한 약리학적 기전에 대해서는 밝혀지지 않고 있다.

본 연구에서는 인간성상세포를 이용하여 熱多寒少湯이 substance P (SP)와 lipopolysaccharide (LPS)에 의해 유도되는 다양한 세포활성물질의 분비량의 조절을 검토함으로써 熱多寒少湯의 약리기전을 면역학적 측면에서 보다 세밀하게 살펴보고자 하였다.

熱多寒少湯 수침액은 인간 뇌 성상세포로 부터 LPS와 SP의 동시자극에 의해 생성되는 세포활성물질중 interleukin (IL)-1, IL-4, IL-6 및 tumor necrosis factor-α (TNF-α)의 분비를 농도의존적으로 억제했다. 그러나 interferon-γ (IFN-γ) 및 IL-2의 분비 조절에는 영향을 미치지 않았다.

그리고 항 IL-1β 항체에 의해 SP 유도성 TNF-α 분비의 증가가 억제되기 때문에 IL-1은 TNF-α 증가를 매개하는 역할을 하는 것으로 사료된다.

이상의 결과는 熱多寒少湯에 의한 급성기 중풍환자 치료 효과가 뇌 성상세포로부터 분비되는 세포활성물질의 조절과 밀접한 관련성이 있다는 것을 암시하고 있다.

Keywords: Yulda-Hanso-Tang, Astrocytes, Tumor necrosis factor-α, Substance P, Lipopolysaccharide, Interleukin-1

I. INTRODUCTION

The Sasang Constitutional medicine classifies people's constitutions into four types, according to the strengths and weaknesses in functions of the internal organs. They are Taeyangin, Taeumin Soyangin and Soumin. Sasang

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Constitutional philosophy forms the basis of treatment by correcting the imbalance of the internal organs caused by the constitutional properties in each body type. For example, Taeumin is thought to have a higher rate of stroke, hypertension and hyperlipidemia than the other types because he or she has a large liver and small lungs. Thus, a person of this body type has a weak dispersing qi and excessive gathering qi[1, 2].

From the viewpoint of the Sasang Constitutional Medicine, Yuldahansotang (YH-Tang) is a prescription which often has been used clinically for neurological diseases, such as Taeumin's stroke and dementia. Recently, many researches support the clinical effect of YH-Tang[3-5]. Among them, Choi[6] has reported that the serum level of cytokine was regulated by YH-Tang in an acute cerebral infarction (CI) of Taeumin.

Astrocytes, one type of the neurological cells, have an important role in maintaining central nervous system (CNS) homeostasis. To carry out homeostatic function, astrocytes synthesize various immune-mediated cytokines and interact with those substances[8-10]. It is known that cytokines are involved in various neuropathological diseases, such as Alzheimer's disease, multiple sclerosis, and acquired immunodeficiency syndrome (AIDS)[11-13]. Also the change of the specific cytokine level was reported in an acute CI patient[14]. Astrocytes were induced to secrete cytokines after interacting with lipopolysaccharide (LPS) or virus.

Substance P (SP) is a neurotransmitter and nerve-originating chemical that mediates neurogenic inflammation[15, 16]. In addition, SP stimulates the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1[17, 18] and IL-6[17], and affects the number of SP receptors when the CNS is injured[19]. SP is widely distributed in the CNS and is believed to stimulate cytokines to influence the pathological process in the CNS[15].

In this study, the regulating effect of YH-Tang on cytokine secretion induced by LPS and SP in astrocytes was investigated. An experiment on the regulating effect of YH-Tang on cytokine secretion in astrocytes was conducted to determine how the regulating mechanism takes place. The author now reports the results which provide the basis for the clinical therapeutic effect on YH-Tang in acute CI patients.

II. MATERIALS AND METHODS

1. Materials

1) Reagents

Fetal bovine serum (FBS), SP, LPS, penicillin/streptomycin, and tween-20 were obtained from Sigma Chemical Co. (Chicago, IL). Cytokines, such as IFN-γ, IL-1, IL-2, IL-4, and IL-6 were obtained from R & D systems (Minneapolis, MN). Human recombinant TNF-α (rTNF-α), polyclonal anti-mouse IL-1α and anti-human TNF-α antibody were obtained from Genzyme (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Life Technologies (Grand Island, NY). Enzyme-linked immunosorbent assay (ELISA) plates were obtained from Nunc (Baperville, IL).

2) Cell line

Human astrocytes, CCF-STTG1 cells were used.

3) Preparation of YH-Tang

The plant sample was obtained from the Kwangju Oriental Medicine Hospital of Wonkwang University. A prescription of YH-Tang weighs 48g, consisting of 16 g of Puerariae Radix (Ge Gen), 8g of Scutellariae Radix (Huang Qin), 8g of Ligustici Tenuissimae Radix (Gao Ben), 4g of Raphani Semen (Lai Fu Zi), 4g of Angelicae Dahuricae Radix (Bai Zhi), 4g of Cimicifugae Rhizoma (Sheng Ma), and 4g of Platycodi Radix (Jie Geng). A water extract of YH-Tang was prepared by decocting the prescription of dried herbs in distilled hot water. The extract was filtered through a 0.45 μm filter and freeze-dried.

The YH-Tang was preserved at 4℃ and used in this study. The yield of the extract was about 10% (w/w).

2. Methods

1) Astrocytes were cultured at 4 × 105 per well and were grown for 3 days with DMEM containing 10% FBS in CO2 incubator.

2) SP preparation:

Special care was taken with SP to avoid possible LPS contamination. First, peptide SP was dissolved in 0.01% acetic acid. Acetic acid was made of 1/10000 glacial acetic acid and filtered through 2.2 μm filter. SP stock solution was kept in a refrigerator at -20℃ after being diluted in LPS non-contaminated distilled water immediately before use.

3) Measurement of cytokines :

Assays of cytokines were performed under modified ELISA according to the procedure outlined by Scuderi et al20). That is, anti-cytokine monoclonal antibody was treated with a coating buffer solution [0.02% sodium azide contained phosphate buffered saline (PBS) pH=7.2] on flat-bottomed 96-well plate (Corning, Rochester, NY) at a final concentration of 6.25 ng in each well, and coated at 4℃ for 12 h. After coating, to reduce non-specific binding, blocking buffered solution, PBS containing 2% BSA, was added and coated at 37℃ for 2 h. Each well was added to 100 μL of recombinant cytokine standard solution and supernatant of each specimen, and after washing 4 times with wash buffer (PBS containing 0.05% Tween 20), were subsequently incubated at 37℃ for 2 h.

The wells were then washed 4 times again with PBS containing 0.05% tween 20. The wells were treated with anti-cytokine antibody which had been diluted by PBS containing 0.05% tween 20, and again incubated at 37℃ for 2 h. After washing repeatedly 7 times with a wash buffer, each well was treated with 100 ng/mL of phosphatase-bound anti IgG antibody (Sigma Co.) and incubated at 37℃ for 2 h, then washed 7 times again. After the last washing step, 100 μℓ of p-nitro phenyl phosphate was added to each well, which was dissolved in buffer solution of the mixture of 0.05M NaHCO3

and 0.05mM MgCl2. Ten min after the color changed, the absorbance of each cytokine was measured at 405 nm wave-length in the ELISA reader. Appropriate specificity controls were included.

3. Statistical analysis

Data are given as means ± S.E. Statistical analysis was performed with Student's t-test. Results with P<0.05 were considered statistically significant.

III. RESULTS

1. The effect of YH-Tang on LPS and SP induced interferon-γ (IFN-γ) secretion in astrocytes

To evaluate the effect of YH-Tang on IFN-γ secretion in astrocytes, the amount of IFN-γ secretion was measured after 18 h of incubation of astrocytes which was added by LPS and SP at various concentration. As shown in Figure 1, YH-Tang did not have an effect on LPS and SP induced IFN-γ secretion in astrocytes.
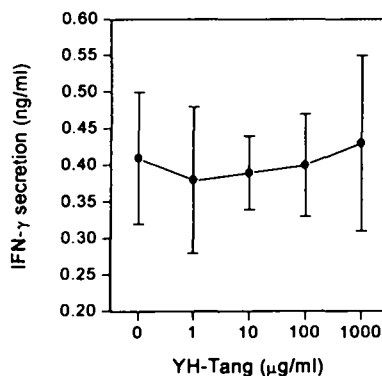

Fig. 1. Effect of YH-Tang on LPS and SP induced IFN-γ secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for IFN-γ. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

2. The effect of YH-Tang on LPS and SP induced IL-1 secretion in astrocytes

The analysis of the effect of YH-Tang on LPS and SP induced IL-1 secretion in astrocytes showed that YH-Tang decreased IL-1 concentration dependently, as shown in Figure 2. The effect of YH-Tang was significant at a concentration of 100-1000 $\mu g/mL$.
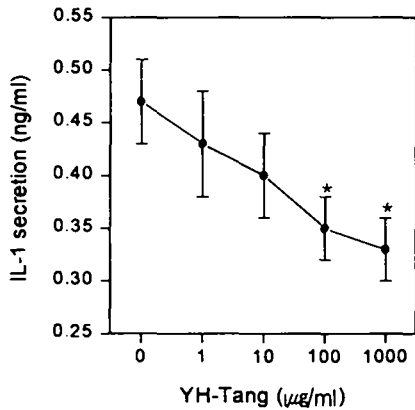
Fig. 2. Effect of YH-Tang on LPS and SP induced IL-1 secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for IL-1. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

3. The effect of YH-Tang on LPS and SP induced IL-2 secretion in astrocytes

As shown in Figure 3, IL-2 secretion was not increased significantly and the regulatory effect of YH-Tang on IL-2 secretion was not significant.
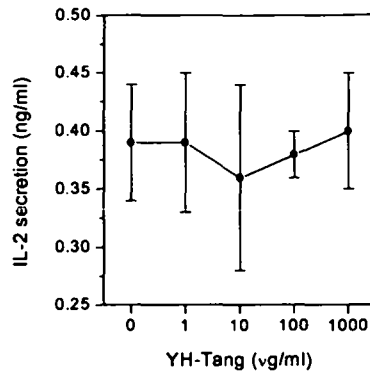
Fig. 3. Effect of YH-Tang on LPS and SP induced IL-2 secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for IL-2. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

4. The effect of YH-Tang on LPS and SP induced IL-4 secretion in astrocytes

The effect of YH-Tang on LPS and SP induced IL-4 secretion in astrocytes was evaluated. As shown in Figure 4, YH-Tang decreased IL-4 concentration dependently.
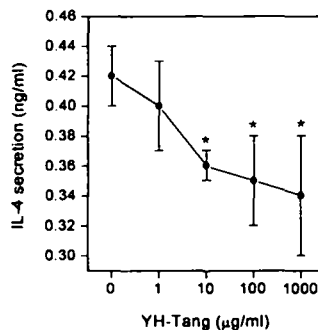
Fig. 4. Effect of YH-Tang on LPS and SP induced IL-4 secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for IL-4. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

5. The effect of YH-Tang on LPS and SP induced IL-6 secretion in astrocytes

The effect of YH-Tang on LPS and SP induced IL-6 secretion in astrocytes was evaluated. As shown in Figure 5, YH-Tang decreased IL-6 concentration dependently.
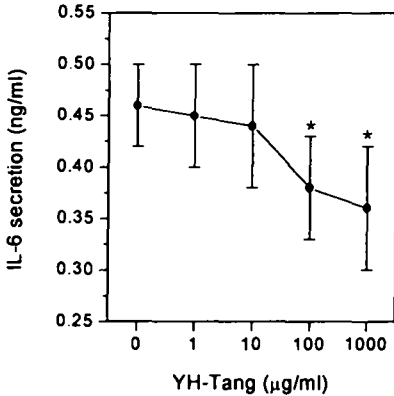

Fig. 5. Effect of YH-Tang on LPS and SP induced IL-6 secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for IL-6. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

6. The effect of YH-Tang on LPS and SP induced TNF-α secretion in astrocytes

The effect of YH-Tang on LPS and SP induced TNF-α secretion in astrocytes was evaluated. TNF-α is an important inflammatory cytokine. TNF-α secretion by astrocytes is induced by LPS and SP. As shown in Figure 6, YH-Tang decreased TNF-α concentration dependently.
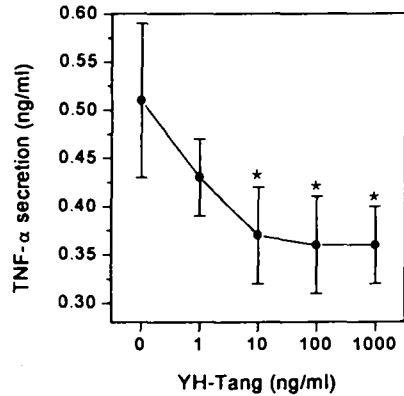

Fig. 6. Effect of YH-Tang on LPS and SP induced TNF-α secretion in astrocytes. The cells (4 × 105 cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of YH-Tang and the supernatants were collected and frozen at -80℃ until assayed for TNF-α. Each datum value indicates the mean ± S.E. of six separated experiments.

*: statistically significant differences from the control values at P < 0.05.

7. The effect of anti IL-1β antibody on LPS and SP induced TNF-α secretion in astrocytes

To evaluate whether YH-Tang's inhibitory effect on TNF-α secretion was mediated via IL-1, the effect of anti IL-1β antibody was assessed. After LPS(1μg/mL) and SP were introduced to an astrocyte incubating solution, anti IL-1β antibody was added and the amount of TNF-α secretion was measured after 24 h. As shown in Figure 7, TNF-α concentration decreased dependently in the anti IL-1β antibody treated group. The inhibitory effect of the anti IL-1β antibody was significant at a concentration of 1-100 μg/mL.
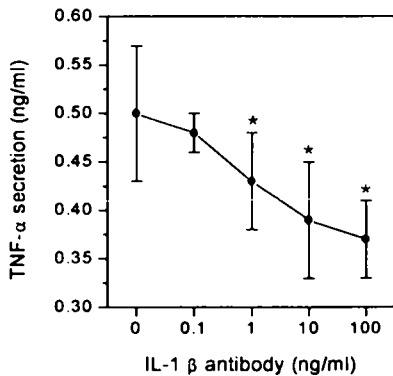
Fig. 7. Effect of anti IL-1β antibody on LPS and SP induced TNF-α secretion in astrocytes. The cells (4 × 10⁵ cells/ml) were incubated for 24 h in medium containing LPS (1 μg/ml) plus SP (2 μg/ml) with various concentrations of anti IL-1β antibody. The supernatants were collected and frozen at -8 0℃ until assayed for TNF-α. Each datum value indicates the mean ± S.E. of four separated experiments.

*: statistically significant differences from the control values at P < 0.05.

IV. DISCUSSION

In this study, the regulatory effect of YH-Tang on LPS and SP induced cytokine secretion in astrocytes was investigated. It has been reported that astrocytes in CNS pathophysiologically are likely to involve in cytokines, both as stimulators and mediators of astrocyte function[10], with which CNS homeostasis is maintianed.

LPS controls many biological properties of gram-negative bacteria. A serological specificity exists in the part of varied polysaccaride and this part belongs to O-antigen of gram-negative bacteria. O-antigen displays a specific receptor of bacteriophage. LPS controls endotoxic activity. If bacteria loses its ability to synthesize LPS, the pathogenicity of bacteria is often lost. The properties of endotoxin include pyrogenicity, lethality, complement activity, B-cell mitogen activity in the mouse, adjuvant acitivity, anti-tumor activity, etc[21].

SP has been reported to induce inflammatory cytokine production in human neuroglial cells and peripheral lymphoid cells as well[22].

It was noted that YH-Tang inhibited the secretion of IL-1, IL-4, IL-6, and TNF-α significantly among the various cytokines produced by simultaneous stimulation of LPS and SP in astrocytes. The effect of YH-Tang was concentration-dependent. It was determined that TNF-α and IL-1 secretion in astrocytes needed LPS stimulation, and LPS stimulation was augmented by SP. Because anti-IL-1β antibody inhibited the secretion of SP-induced TNF-α, IL-1 was considered as a mediator of TNF-α increase. These results are the evidence that SP, a neurotransmitter produced in the nerve of the CNS, is an important molecule involved in inflammation. IL-1 is an endogenous pyrogen, the activating factor of lymphocyte products. It is made in many cells, especially macrophage cells, where it is made most. IL-1 activates T-cell and B-cell, and causes the inflammatory response. In the brain, IL-1 causes heat and increases the secretion of corticosteroid[23].

According to Touzani et al's recent report[24], IL-1 has an important role in the development of brain damage following cerebral ischemia. The expression of IL-1 in the brain is dramatically increased during the early and chronic stages of infarction. The most direct evidence that IL-1 contributes significantly to ischemic injury is that (1) central administration of IL-1β exacerbates brain damage, and (2) injection or over- expression of IL-1 receptor antagonist, and blockade of IL-1β converting enzyme activity dramatically reduce infarction and improve behavioral deficit. The mechanisms underlying IL-1 actions in stroke have not been clearly elucidated, and it seems likely that its effects are mediated through stimulation and inhibition of a wide range of pathophysiological processes.

According to Yamasaki et al's report[25], the contribution of cytokines in an inflammatory cascade on cerebral reperfusion injury are characterized as consisting of typical phases; leukocytes invasion, microglial activation and remodeling. Within 1-2 days, IL-1 and TNF-α induce the expression of adhesion molecules that cause leukocytes to adhere to endothelial cells.

Barone et al[26] have reported that TNF-α is a pleiotypic cytokine that rapidly upregulates in the brain after injury and blocking endogenous TNF-α also significantly reduced focal ischemic brain injury and especially, treatment with 60 pmol monoclonal antibody before and after permanent middle cerebral artery occlusion significantly reduced infarct size compared with control (non-immune) antibody treatment.

Ali et al[27] have reported that in the brain, the expression of the pleiotropic cytokine IL-6 is enhanced in various chronic or acute CNS disorders and focal cerebral ischemia in rats early up-regulated the expression of IL-6 mRNA. Additionally, the striatal injection of N-methyl-D-aspartate in rats, a paradigm of excitotoxic injury activated the expression of IL-6 mRNA.

IL-4, which is secreted in activated Th2-cell and mast cell, helps to increase B-cell. It also increases the induction of Th2-cell, which regulates the amount and activity of eosinophil and mast cell. Therefore, if IL-4 is overgenerated, IgE is increased, thus causing allergy[23]. In other words, the role of IL-4 in the regulation of IgE has been assessed in a number of disorders associated with increased IgE production. Our finding of elevated IL-4 levels in patients with CI could be related to the high IgE levels found in allergic diseases. Thus, further elucidation on the roles of IL-4 and IgE should facilitate the development of a novel therapy or preventive measures against CI[6].

The Sasang Constitutional Medicine was established by Je-Ma Lee of Korea in 1894. In Sasang Constitutional Medicine, the symptoms of diseases in each constitutional type are classified according to exterior and interior diseases. Sasang Constitutional Medicine forms the basis of treatment by harmonizing the interrelationship between the internal organs, thereby recovering the body's homeostatis and enabling the body to prevent disease. In other words, if the human body maintains a balanced and harmonious states, its immune system will be working at an optimal level and the body's ability to prevent or fight off most diseases will be strengthened. Therefore, we can enhance the body's ability to prevent disease and to recuperate from disease damage by promoting beneficial immune responses and repressing the harmful ones.

The Sasang Constitutional medical approach to disease is distinctive in that it focuses on endogenous etiological factors rather than exogenous ones. Even in diseases caused by exogenous etiological factors it focuses on natural treatment through self recovery of body's immune function by remedying endogenous etiological factors[28].

YH-Tang is based on this Sasang Constitutional medical thought and is used in interior diseases of Taeumins. It is composed of Puerariae Radix (Ge Gen), Scutellariae Radix (Huang Qin), Ligustici Tenuissimae Radix (Gao Ben), Raphani Semen (Lai Fu Zi), Angelicae Dahuricae Radix (Bai Zhi), Cimicifugae Rhizoma (Sheng Ma), and Platycodi Radix (Jie Geng)[29].

Recently from the viewpoint of Sasang Constitutional Medicine, the early symptoms of stroke are regarded as a part of the category of interior diseases, and such view is actively applied to a treatment.

YH-Tang is a prescription which is used to treat stroke of Taeumins, but its exact pharmacological mechanism is not well understood. Recently many researches are being conducted to investigate the YH-Tang's effect on the treatment of stroke. Among them, Choi[6] reported on the effect of YH-Tang on the regulation of cytokines in a Taeumin CI patient. This discovery provides an important indication that pathological changes in an acute stroke patient can be recognized by a specific cytokine measurement.

It has been reported that especially IL-1, TNF-α, IL-6 are closely associated with brain damage. However, the direct mechanism of IL-4 has yet to be clarified. The immunological relation between CI and allergy is assumed.

Though astrocytes of a Taeumin were not used, it was confirmed that YH-Tang influences the regulation of specific cytokine secretion in astrocytes. The fact that YH-Tang significantly inhibits the production of inflammatory cytokines, such as IL-1, IL-6, TNF-α in

astroyctes, and IL-4 in Th2 cells supports Choi's report.[6] Therefore, YH-Tang is thought to be applicable to the treatment of the acute CI. Nevertheless, the exact mechanism and effect of YH-Tang at a molecular level should be established in the future.

V. CONCLUSIONS

The author has obtained the following results through the study of YH-Tang's effect on the regulation of various cytokines in human astrocytes

1. In human astrocytes, YH-Tang inhibited IL-1, IL-4, IL-6 and TNF-α secretion induced by LPS and SP concentration-dependently.
2. However, YH-Tang did not have an effect on IFN-γ and IL-2 secretion.
3. It was confirmed that TNF-α secretion, which was stimulated simultaneously by LPS and SP, was mediated by IL-1.

The results suggest that the regulation of cytokine secretion in astrocytes is closely involved in the therapeutic effect of YH-Tangin on acute stroke patients.

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