Effect of Herbal Therapy on Intracellular Cytokine Expression of CD8 Cell in Nasopharingeal Cancer Patients

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Diterima tanggal, 16 Maret 2011, Disetujui 21 Maret 2011

ABSTRACT

Aims: to know the effect of THL medicinal herb on the immune response of nasopharyngeal cancer (NPC) patients by measuring the intracellular cytokine level (IFN-γ and TNF-α) before and after THL administration. To introduce the intercellular cytokine evaluation method for evaluating immune response.

Methods: fifteen patients with nasopharyngeal cancer were included in this study and received Tien Hsien Liquid (THL) four times a day for 4 weeks. Before and after THL treatment, 10 mL blood sample were taken to measure intracellular cytokine (IFN-γ and TNF-α) both spontaneous and stimulated by phytohemaglutinin (PHA). The IFN-γ and TNF-α were measured by flowcytometric assay.

Result: Both spontaneous and stimulated intracellular cytokine were increased after THL treatment. Percentages of differences of spontaneous IFN-γ and TNF-α were 4.62±1.39 and 4.89±1.39; whereas for stimulated IFN-γ and TNF-α were 3.98±1.29 and 1.65±3.82.

Intracellular IFN and TNF-α evaluation can be performed in our laboratory as an alternative evaluation of IFN and TNF-α serum level. In the IFN and TNF-α serum evaluation, the measured of IFN and TNF-α level were produced by many cell types such as macrophages, endothelial cells and others while with the intracellular method that we used in this report we measured the proteins that produced by CD8 cells.

Conclusion: THL can modulate the cellular immune response by increasing the intracellular cytokine (IFN-γ and TNF-α) in $CD8^+$ cell suggesting that this herb may be a potential immunoceutical agent to be used as supportive agent for the treatment of cancer especially nasopharyngeal cancer.

***Key Words**: intracellular cytokine, nasopharyngeal cancer, Tien Hsien Liquid (THL)*

ABSTRAK

Tujuan: untuk mengetahui efek obat herbal terhadap respons imun pasien kanker nasofaring (KNF) dengan cara mengukur persentase sitokin intraselular (IFN-γ dan TNF-α) sebelum dan sesudah pemberian THL. Juga untuk memperkenalkan metode pemeriksan sitokin intra sel sebagai alternatif lain dalam penilaian respons imun pasien.

Metode: sebanyak 15 pasien KNF diikutsertakan secara konsekutif dalam penelitian ini dan menerima obat Tien Hsien Liquid (THL) sebanyak empat kali sehari selama empat minggu. Sebelum dan sesudah pemberian THL, dilakukan pengambilan darah sebanyak 10 mL untuk pengukuran kadar sitokin intraselular TNF-α dan IFN-γ, baik secara spontan maupun setelah perangsangan *phytohemagglutinin* (PHA) dengan menggunakan *flowcytometric assay*.

Hasil: kedua jenis sitokin (TNF-α dan IFN-γ) baik sebelum maupun setelah perangsangan PHA mengalami kenaikan setelah pemberian THL. Persentase kenaikan IFN-γ dan TNF-α sebelum perangsangan PHA adalah 4.62±1.39 dan 4.89±1.39; sedangkan persentase kenaikan setelah perangsangan PHA adalah 3.98±1.29 and 1.65±3.82.

Pada laporan ini juga diperlihatkan bahwa pemeriksaan sitokin intra sel dapat dilakukan di laboratorium kami sebagai alternatif lain penilaian respons imun pasien. Pada pemeriksaan TNF-α and IFN dalam serum, kadar TNF-α and IFN yang diukur diproduksi oleh berbagai sel lain seperti makrofag, sel endotel, dan lain-lain, sedangkan dengan metode pemeriksaan sitokin intraseluler yang dikerjakan dalam laporan ini yang diukur adalah kadar protein yang diproduksi sel CD8.

Kesimpulan: THL dapat memodulasi respons imun selular dengan meningkatkan sitokin intraselular (IFN-γ dan TNF-α) dalam $CD8^+$. Oleh karena itu, obat herbal ini dapat menjadi agen *immunoceutical* yang dapat digunakan sebagai terapi suportif pada pasien kanker, terutama pasien kanker nasofaring (KNF).

Kata kunci: sitokin intraselular, kanker nasofaring, Tien Hsien Liquid (THL)

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INTRODUCTION

Immune response to neoplasm occurred especially as cellular immune response, mediated by T cell mostly $CD8^+$ cell.[1] They recognize antigen presented by the antigen presenting cell (APC) and destroy cell that contain virus, malignant cell, or hystocompatible cell.[2,3] Cellular immune response needs cytokine to mediate its work.[4] Karanikas et al found that cytokine level of Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interferon γ (IFN-γ) and *tumor necrosis factor* α (TNF-α) are activated by T cell lymphocyte.[5]

Cytokines are soluble protein that have important role in the growth regulation and differentiation as well as function in a wide range of cells. Recent research have shown that cytokines have multiple functions, target a wide range of cells and can be expressed by diverse cellular subsets, among others lymphocytes. Previous studies have shown the correlation between lymphocytes with certain cytokines for example TH-1 with IL-2, interferon γ, and TH-2 with IL-4, IL-5 and IL-10.[6]

The ability to assess intracellular cytokine within a single cell has become a potent tool.[7] Two methods to detect intracellular cytokines have been reported. The first method was reported by Sanders in 1991 who reported a method to detect intracellular cytokines by a microscopic method using parafomaldehyde fixation, saponin permeabilization and indirect immunofloure-cense staining. The second method was reported by Yung in 1993 who stimulated cells in the presence of Monensin which disrupt intracellular protein transport and causes accumulation of intracellular cytokines inside the Golgi apparatus. Because of peripheral blood T lymphocyte cells do not produce or produce only small amount of cytokines spontaneously, these cells must first be stimulated.[8,9] Several stimulators have been known such as Phorbol Myrisate Acetat (PMA), phytohemaglutinin (PHA), and ionomycine.[10] PMA and ionomycin are commonly used as stimulant, but these agents lead to rapid down regulation.[11]

Currently, there are many research studies concerning the role of various cytokines in the pathogenesis of various illnesses including cancer. Immune deficiency in cancer patients including nasopharyngeal cancer results in the invasion of the tumor cells.[12-15] Nasopharyngeal carcinoma (NPC) is an epithelial cancer which associated with Epstein-Barr virus (EBV) infection.[16,17] In Dharmais Cancer Hospital, NPC is one of the top five most frequent cancer diagnosed in 2007.[18]

Tien-Hsien Liquid (THL) that is produced by Feida *Union Pharmaceutical Manufactory* is extracted from fourteen Chinese medicinal herbs and has been used for more than 10 years as a supplement for cancer. Some studies demonstrated that THL have a strong immunomodulating effect and a strong anti-cancer activity.[19-22] THL has been used in several cancer cell lines such as breast cancer, cervical cancer, hepatoma, lung cancer, prostate cancer and nasopharyngeal cancer.[21,22] Previous studies reported that THL has immunomodulator effect in patients with recurrent aphtous ulcerations.[19,20] The other study by Sun et al reported that THL is safe and has no side effect. Moreover, THL also has apoptosis effect in cancer cell but not in normal cell.[21]

In this study, we want to know the effects of THL on cellular immune response of nasopharyngeal cancer patients by measuring the level of intracellular cytokine (IFN-γ and TNF-α) by using PHA as a stimulating agent.

METHODS

Subjects

Fifteen patients with nasopharyngeal cancer were included in this study consecutively in the period between August 2007 and August 2008. All the subjects diagnosed as a nasopharyngeal cancer regardless of stage and histology type of the cell. Those who had not received chemotherapy or more than one month after receiving chemotherapy/radiotherapy and agree to follow the study by signing the informed consent were also included. Patients with sign of infection or pregnant woman were excluded from this study. All the patients were diagnosed and treated in the Dharmais National Cancer Center, Jakarta, Indonesia. After the patients signed informed consent, 10 mL blood sample were taken and they would receive THL four time a day for 4 weeks. After one week, 10 mL blood sample would be taken again. This study has been approved by the Ethical Committee of the Dharmais Cancer Center Hospital.

Cell Preparation and Intracellular Cytokine Assay

Peripheral blood samples were collected from all of the subjects into heparin anti coagulant. Peripheral blood mononuclear cells (PBMCs) and lymphocytes were isolated from blood samples by Ficoll-Paque centrifugation. After centrifugation, PBMC ($1x10^6$ cells/mL) were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum.

The cells used in this study were isolated lymphocytes and PBMC collected before and after consumption of the medicine for 2 weeks. Spontaneous intracellular cytokine expression was measured after cells were cultured for 24 hours.

Intracellular cytokine expression of activated lymphocytes is investigated by measuring it in lymphocytes which have been stimulated by PHA (50 ng/mL). No serum added to the cultures. After were washed, the lymphocytes and PBMC were permebialized and fixed using Ortho Permeafix (Ortho Diagnostics Systems, Inc., Raritan, N.J.).

After permeabilization and fixation, all staining was done at room temperature for 30 minutes in the dark. Staining was followed by a buffered saline wash. Dual-color and triple color cytometry was done by using a FACSort or FACSCalibur flow cytometer and CellQuest software according to the manufacturers' instruction (BD/PMG and Genzyme Diagnostics, Cambridge, Mass).

Statistical Analysis

We used univariate analysis to describe clinicopathological data. Paired sample t test was used to compare level of cytokine intracellular before and after THL treatment. The result was considered significant if the p value was less than 0.05. In this report, we use the SPSS program in the data analysis.

RESULTS

A total of fifteen nasopharyngeal cancer patients who received Tien Hsien Liquid (THL) were studied, with age ranging 19 to 62 years old and with the mean 36.2 years old. Detailed data about clinicopathology can be seen in table 1.

Table 1: Clinicopathology data

Clinicopathology	Frequency	Percentage (%)
Histology		
Undifferential carcinoma	14	93.3
Squamous cell carcinoma	1	6.7
Stage		
II	2	13.3
III	5	33.3
IVA	3	20.0
IVB	1	6.7
IVC	4	26.7

Table 2: Intracellular cytokines before and after THL treatment

	Before THL (%)	After THL (%) Differences ± SEM	Mean of	p
Spontaneous				
IFN	6.76±9.37	11.38±10.19	4.62±1.39	0.005
TNF	6.64±9.33	11.53±9.30	4.89±1.39	0.003
Stimulated by PHA				
IFN	6.99±8.20	10.98±11.24	3.98±1.29	0.008
TNF	7.66±13.15	9.31±9.83	1.65±3.82	0.672

DISCUSSION

Cytotoxic T cell (CD8) plays an important role during the body cellular immune response to cancer cells. Activated CD8 cells produce interferon and interleukin during performing cellular immune response. Since interferon and interleukin are also excreted by other cells the technique to measure the interferon and interleukin expressed intra CD8 cells (intra cellular) is very important.

Besides, the measurement of cytokine levels has yielded useful information on the pathologic process in various disease such as inflammatory disease (crohn's disease, rheumatoid disease), allergy (asthma, atopic dermatitis), infection (tuberculosis) and also in cancer.[23-28]

In this study we described the method for detecting intracellular cytokines in human PBMCs. By the use of three-color flowcytometri analysis we were able to define precisely the population of lymphocytes and monocytes and to associate the cell phenotypes with the production of certain cytokines. We compared the un-stimulated and stimulated intracellular cytokines by PHA. We found that both of spontaneous and stimulated intracellular cytokine were increase after THL treatment, which spontaneous cytokine was higher than stimulated. This founded was not similar with other studies.[9,29] Sullivan found that combination PHA and PMA is the potent stimulant.[9] Whereas Baran study found that TNF α and INF γ was higher when stimulated by combination PMA and ionomycin and incubated in 6 hours.[29] Thus, the protocol to measure intracellular cytokine should be more improved with emphasize to type of stimulants and duration of incubation. It is hoped that this technique can be used as an additional test to study other immunomodulators

More pharmaceutical companies introduce immunomodulators to medical professions lately. Most of these new products are herbal products. Nevertheless only a few herbs product already studied whether they have immune modulation effect. THL is one of herbs product that has been studied having ability to modulate immune response, but the study limited in chronic inflammatory disease such as aphtous and lichen planus.[19,20] This herb also has been studied in cancer but the point of interest tends to the effect of apoptosis, anti metastasis and anti angiogenesis.[21,22] Thus, this study is a pioneer in studying THL as an immunomodulator in cancer especially NPC. This study proved that THL can increase the cytokines within $CD8^+$ cell in NPC patients. Further study is needed especially to learn the effect of THL given to other cancers patients. Moreover, the effect of THL that is given to patients with post chemotherapy or between chemotherapy cycles can be studied. Since chemotherapy can decrease the immune system and increase risk of infection, it can learn more about the role of THL in the occurrence of infection in cancer patients with chemotherapy.

CONCLUSION

This study suggest that the studied herbal extract (THL) can modulate the cellular immune response by

increasing the intracellular cytokine (IFN-γ and TNF-α). Therefore, it may be a potential immunomodulator (immunoceutical) agent for the treatment cancer especially nasopharyngeal cancer, ◆

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