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Immunomodulatory function of orally administered thymosin α1*

CHEN Xiang-ming (陈祥明)[1], JIANG Han-liang (蒋汉梁)[2], ZHOU Lin-fu (周林福)[2], PAN Xiao-ping (潘小平)[2], HU Zhong-rong (胡中荣)[2], LIU Rong-hua (刘荣华)[2], CHEN Xiao-ming (陈晓明)[2], CHEN Zhi (陈 智)†‡[2]

([1]*Department of Anesthesiology, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)
([2]*Institute of Infectious Diseases, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)
†E-mail: chenzhi@zju.edu.cn

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Abstract: Objective: To investigate the immunological function of a yeast expression system for thymosin α1 (Tα1). Methods: A constructed Tα1 yeast expression system was used to investigate the immunological function of orally administered Tα1. Dried yeast containing three different concentration of Tα1 was fed to normal Balb/c mice and other Balb/c mice whose immunities were inhibited in advance by cyclophosphamide. Synthesized Tα1 peptide was used as positive control and dried yeast with empty plasmid was used as negative control. $CD4^+$ and $CD8^+$ levels were detected by flow cytometry assay. TNF-α, IFN-γ, IL-2, IL-6 and IL-10 levels were detected by liquid chip. Results: In normal Balb/c mice or immune inhibition Balb/c mice, $CD8^+$ levels were significantly increased. Especially in immune inhibition Balb/c mice, $CD8^+$ levels in synthesized Tα1 group (18.77%±4.72%), small dose group (13.48%±6.17%) and large dose group (22.74%±1.09%) were significantly higher than that in empty yeast control group (7.49%±2.14%). Conclusion: Orally administered Tα1 has its certain immunomodulatory function.

Key words: Thymosin α1, Yeast expression system, Biological activity, Orally administered
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INTRODUCTION

The thymus is a vital immune organ and plays a very important role in the development and maintenance of the lymph system. The extract of animal thymus has been used in clinical practice as an adjunct treatment in patients with carcinoma, viral infection and immunodeficiency, but the extract containing miscellaneous animal proteins may easily lead to allergy in patients. Thymosin α1 (Tα1) is the most potent ingredient in thymosin and the biological activity of pure Tα1 is 10~1000 times that of total thymus extract. Pure chemically synthesized Tα1 has already been used as an immune-mediating factor in clinical practice, but high price and inconvenient intramuscular injection administration limit its wide use. A new approach is needed to produce high quality, cheap and orally administered Tα1 to satisfy clinical practice. In this study, a constructed Tα1 yeast expression system was used to investigate the immunological function of orally administered Tα1.

MATERIALS AND METHODS

Construction of Tα1 yeast expression system

The whole Tα1 gene was obtained by PCR with upstream primer (5′ GGA AGC TTT CTG ATG CTG CTG TTG 3′ containing *Hin*d III restriction enzymatic site) and downstream primer (5′ TAT GGA TCC TAG TTC TCA GCC TCT TCG 3′ containing *Bam*H I restriction enzymatic site). After digestion with *Hin*d III and *Bam*H I restriction enzymes, Tα1 gene was cloned into pYES2 plasmid (ClonTech

‡ Corresponding author
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Corporation, Cat. V825-20). The positive clones were identified by PCR, restriction enzyme digestion, sequencing and Western blot, respectively. Synthesized Tα1 peptides (Ac-SDAAVDTSSEITTKDLKEKKE VVEEAEN-OH, synthesized by Xian Meilian Company) were used as positive control.

Construction of immune inhibition Balb/c mouse model

Forty-six Balb/c mice were injected intra-peritoneally with cyclophosphamide (Jiangsu Henrui Pharmaceutical Corporation, Batch NO. 01101021) (100 mg/kg) in the 1st and 2nd day, and the other 6 Balb/c mice were injected intra-peritoneally with normal saline at the same volume as normal control. On the 8th day, blood was drawn from orbit of 6 Balb/c mice selected randomly from cyclophosphamide treated group and 6 Balb/c mice of normal control under anesthesia with ether. Flow cytometry assay was used to analyze $CD4^+$ and $CD8^+$ lymphocytes.

Treatment with Tα1 expressed by yeast

The remaining 40 Balb/c mice treated with cyclophosphamide were divided into 5 groups randomly and evenly: Empty yeast control group was fed with yeast including empty pYES2 (containing 30 μg of lyophiled INVSc1 yeast including empty pYES2 plasmids in 0.1 ml normal saline) through feeding tube, the synthesized Tα1 group received intra-peritoneal injection with synthesized Tα1 peptide (containing 6 μg synthesized Tα1 peptides in 0.1 ml normal saline), and small dose group, medium dose group and large dose group received pYES2-Tα1 treatment through feeding tube at low, medium or high concentration (containing 10 μg, 30 μg, 60 μg of lyophiled INVSc1 yeast including pYES2-Tα1 plasmids in 0.1 ml normal saline, respectively). Each mouse received treatment everyday from 9th day. Blood was drawn from orbit under anesthesia on the 15th day for the detection of Tα1 effect.

Forty normal mice were also used to investigate the effect of Tα1 with the same method of grouping and treatment as immune inhibition mice mentioned above.

Flow cytometry assay

Flow cytometry assay was used to determine percentages and absolute numbers of $CD4^+$ and $CD8^+$ lymphocytes.

Liquid chip assay for cytokines

Cytokines were detected by LabMAP^{TM} system (Laboratory Mutli-Analyte Profiling, Luminex, Austin, TX) with LiquiChip^{TM} Mouse 10-Cytokine Kit (Qiagen Corporation, Cat. No. 922028). Briefly, serial dilutions of the standard dilution were prepared and samples were put into the relevant well of the filter plate. Then bead suspension added to each well was vortexed and incubated for 2 h. After washing the mixture twice with LiquiChip Cytokine Assay Buffer, LiquiChip Mouse 10-Cytokine Antibody was added and the mixture was incubated for 90 min. Then diluted streptavidin-phycoerythrin was added and the mixture was incubated for 30 min. After reaction stopped, the plate was assayed on a LiquiChip Reader using the Mouse Cytokine Assay system. In order to minimize the effect of individual differences, the same volume samples in the same group were mixed completely, and each mixed sample was detected twice by LabMAP^{TM} system.

Statistical analysis

All the data were analyzed with SPSS 11.0 software and student *t*-test was used. A P value less than 0.05 indicated statistically significant difference.

RESULTS

Identification

Tα1 yeast expression system was identified by PCR, restriction enzyme, sequencing and Western blot, respectively. The results showed that the positive clone had correct sequence and expressed correct Tα1 protein (data not shown).

The immune inhibition effect of cyclophosphamide on Balb/c mice

$CD4^+$ and $CD8^+$ lymphocytes in those Balb/c mice treated with cyclophosphamide were significantly lower than those in normal ones (32.50%±10.76% vs 57.14%±9.58%, $P<0.01$, and 12.29%±3.49% vs 20.14%±5.53%, $P<0.05$), which showed that the cellular immune function was significantly inhibited. The immune inhibition model had been constructed successfully (Table 1).

Effect of Tα1 expressed by yeast on normal mice

As showed in Table 2, compared with normal control, although small dose and large dose of Tα1 expressed by yeast improved the $CD8^+$ lymphocytes (19.06%±5.94%, 18.04%±4.76% vs 11.05%±0.85%, P<0.01), both of them remained in normal level. There were no significant changes in other groups.

Effect of Tα1 expressed by yeast on immune inhibition mice

As showed in Table 3, compared with empty yeast control, small dose group, large dose group and positive control group significantly improved the $CD8^+$ lymphocytes (13.48%±6.17%, 22.74%±1.09% and 18.77%±4.72% vs 7.49%±2.14%, P<0.01), while all of them had little effect on the $CD4^+$ lymphocytes (63.62%±8.19%, 61.86%±6.94% and 65.91%±4.78% vs 57.93%±10.40%, P>0.05).

Liquid chip for cytokine

The results (Fig.1) showed that compared with empty yeast, synthesized Tα1 and Tα1 expressed by yeast could improve the levels of TNF-α, IL-6 and IL-10 significantly, and that synthesized Tα1 can improve the IFN-γ and IL-2 levels as well.

DISCUSSION

Tα1 plays many vital roles, such as immune regulation, promoting NK cell activity, enhancing the anti-infection ability of the host, promoting viral clearance, anti-oxidant, inhibiting the growth of cancer cells, etc. (Zavaglia *et al.*, 2000). It was used widely in clinical treatment of hepatitis B, hepatitis C, HIV and some tumors, such as melanoma, lung cancer, leukemia, squamous epithelial cancer, colon cancer, etc. (Kullavanuaya *et al.*, 2001; Andreone *et al.*, 2001; Garaci *et al.*, 2000； Saruc *et al.*, 2003).

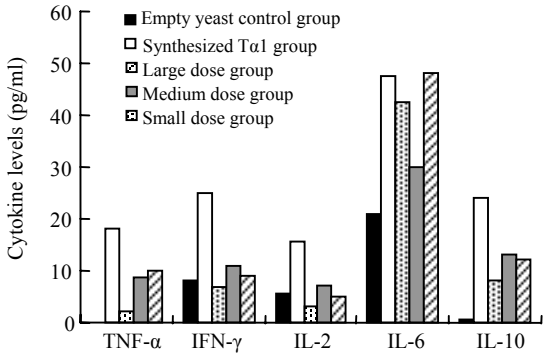

Fig.1 The effect of Tα1 on several cytokines in immune inhibition Balb/c mice

Table 1 Effect of cyclophosphamide on the immune function of Balb/c mice

Group	Number	Cyclophosphamide dose (mg/kg)	$CD4^+$ (%)	$CD8^+$ (%)
Normal group	6	0	57.14±9.58	20.14±5.53
Immune inhibition group	6	100	32.50±10.76**	12.29±3.49*

**Compared with normal group, P<0.01; *Compared with normal group, P<0.05

Table 2 The effect of Tα1 on immune function of normal Balb/c mice

Group	Animal numbers	Dose (μg/mouse)	$CD4^+$ level (%)	$CD8^+$ level (%)
Empty yeast control group	8	30	55.32±3.95	11.05±0.85
Synthesized Tα1 group	8	6	56.10±11.09	11.79±2.52
Small dose group	8	10	53.01±17.98	19.06±5.94**
Medium dose group	8	30	59.25±13.69	12.72±2.81
Large dose group	8	60	62.84±6.00	18.04±4.76**

**Compared with empty yeast control group, P<0.01

Table 3 The effect of Tα1 on immune function of immune inhibition Balb/c mice

Group	Animal numbers	Dose (μg/mouse)	$CD4^+$ level (%)	$CD8^+$ level (%)
Empty yeast control group	8	30	57.93±10.40	7.49±2.14
Synthesized Tα1 group	8	6	65.91±4.78	18.77±4.72**
Small dose group	8	10	63.62±8.19	13.48±6.17**
Medium dose group	8	30	58.48±9.46	8.71±1.72
Large dose group	8	60	61.86±6.94	22.74±1.09**

**Compared with empty yeast control group, P<0.01

There are two kinds of thymus products for clinical practice: One is Zadaxin. It has good effect in clinical practice, but one patient has to spend $8000 in one period of treatment; the other produced domestically, is thymosin mixture extracted from animal thymus. It has poorer curative effect (only 0.56%~1.00%). What is more, it is mixed with animal proteins and easily induces allergy in patients, so it cannot be widely used in clinical practice. It is vital to construct a new system, which can produce Tα1 by gene engineering and have merits of low cost and high quality.

The results of this study showed Tα1 expressed by yeast can be administered orally. Tα1 expressed by this system can modulate the immune function of normal Balb/c mice and Balb/c mice treated with cyclophosphamide in advance. The $CD8^+$ lymphocytes were significantly increased.

The LiquiChip Mouse 10-Cytokine Kit enables simultaneous analysis of several mouse cytokines, such as TNF-α, IFN-γ, IL-2, IL-6 and IL-10, in a rapid, homogeneous, bead-based assay. LiquiChip assays measure the interaction of immobilized, bead-bound assay components with reaction partners in solution. In the present study, we found that compared with empty yeast, synthesized Tα1 and Tα1 expressed by yeast can improve the levels of TNF-α, IL-6 and IL-10 significantly. But as with IFN-γ and IL-2, the increases were only observed in the synthesized Tα1 group. Why did Tα1 expressed by yeast have little effect on these two cytokines? Was it related to the way of administration of Tα1 or was it just related to the insufficient dose of Tα1 in the yeast expression system? This deserves further investigation.

In short, this study showed that the Tα1 expressed by yeast expression system had its certain immunomodulatory function.

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