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Immunomodulatory function of orally administered thymosin $\alpha 1$ ^{*}

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Abstract: Objective: To investigate the immunological function of a yeast expression system for thymosin $\alpha 1$ (Ta1). Methods: A constructed Ta1 yeast expression system was used to investigate the immunological function of orally administered Ta1. Dried yeast containing three different concentration of Ta1 was fed to normal Balb/c mice and other Balb/c mice whose immunities were inhibited in advance by cyclophosphamide. Synthesized Ta1 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 Ta1 group (18.77% $\pm$ 4.72%), small dose group (13.48% $\pm$ 6.17%) and large dose group (22.74% $\pm$ 1.09%) were significantly higher than that in empty yeast control group (7.49% $\pm$ 2.14%). Conclusion: Orally administered Ta1 has its certain immunomodulatory function.

Key words: Thymosin $\alpha 1$, 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 $\alpha 1$ (Ta1) is the most potent ingredient in thymosin and the biological activity of pure Ta1 is 10~1000 times that of total thymus extract. Pure chemically synthesized Ta1 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 Ta1 to satisfy clinical practice. In this study, a constructed Ta1 yeast expression system was used to investigate the immunological function of orally administered Ta1.

MATERIALS AND METHODS

Construction of Ta1 yeast expression system

The whole Ta1 gene was obtained by PCR with upstream primer (5' GGA AGC TTT CTG ATG CTG CTG TTG 3' containing *Hind* 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 *Hind* III and *Bam*H I restriction enzymes, Ta1 gene was cloned into pYES2 plasmid (ClonTech

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Corporation, Cat. V825-20). The positive clones were identified by PCR, restriction enzyme digestion, sequencing and Western blot, respectively. Synthesized T α 1 peptides (Ac-SDAAVDTSSSEITTKDLKEKKE 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 T α 1 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 μ g of lyophilized INVSc1 yeast including empty pYES2 plasmids in 0.1 ml normal saline) through feeding tube, the synthesized T α 1 group received intra-peritoneal injection with synthesized T α 1 peptide (containing 6 μ g synthesized T α 1 peptides in 0.1 ml normal saline), and small dose group, medium dose group and large dose group received pYES2-T α 1 treatment through feeding tube at low, medium or high concentration (containing 10 μ g, 30 μ g, 60 μ g of lyophilized INVSc1 yeast including pYES2-T α 1 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 T α 1 effect.

Forty normal mice were also used to investigate the effect of T α 1 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 LabMAPTM system (Laboratory Mutli-Analyte Profiling, Luminex, Austin, TX) with LiquiChipTM 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 LabMAPTM 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

T α 1 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 T α 1 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% $\pm$ 10.76% vs 57.14% $\pm$ 9.58%, *P*<0.01, and 12.29% $\pm$ 3.49% vs 20.14% $\pm$ 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 T α 1 by gene engineering and have merits of low cost and high quality.

The results of this study showed T α 1 expressed by yeast can be administered orally. T α 1 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 T α 1 and T α 1 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 T α 1 group. Why did T α 1 expressed by yeast have little

effect on these two cytokines? Was it related to the way of administration of T α 1 or was it just related to the insufficient dose of T α 1 in the yeast expression system? This deserves further investigation.

In short, this study showed that the T α 1 expressed by yeast expression system had its certain immunomodulatory function.

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