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Review

Thymosin alpha 1: Biological activities, applications and genetic engineering production

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ABSTRACT

Thymosin alpha 1 (Tα1), a 28-amino acid peptide, was first described and characterized from calf thymuses in 1977. This peptide can enhance T-cell, dendritic cell (DC) and antibody responses, modulate cytokines and chemokines production and block steroid-induced apoptosis of thymocytes. Due to its pleiotropic biological activities, Tα1 has gained increasing interest in recent years and has been used for the treatment of various diseases in clinic. Accordingly, there is an increasing need for the production of this peptide. So far, Tα1 used in clinic is synthesized using solid phase peptide synthesis. Here, we summarize the genetic engineering methods to produce Tα1 using prokaryotic or eukaryotic expression systems. The effectiveness of these biological products in increasing the secretion of cytokines and in promoting lymphocyte proliferation were investigated *in vitro* studies. This opens the possibility for biotechnological production of Tα1 for the research and clinical applications.

Contents

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1. Introduction

Thymosin alpha 1 (Tα1), a biologically active peptide consisting of 28 amino acid residues, was first described and characterized by Goldstein et al. [34]. The research process of Tα1 began with the study on the thymus, which is an important vital organ for homeostatic maintenance of peripheral immune system [48]. In 1966, Goldstein et al. [35] first isolated and described a lymphocytopoietic factor from calf thymus, which was termed "thymosin". The multiple action of thymosin on the immune, endocrine and central nervous systems was revised by Goldstein and Badamchian [32]. Further purification of this factor led to the isolation of a heat-stable acetone-insoluble preparation, termed thymosin fraction 5 (TF5), which could induce T cell differentiation, enhance immunological function [36] and induce apoptosis of neuroendocrine tumor cells [72]. The promising results seen with TF5 provided the scientific rationale to further isolate and characterize the molecules in TF5 responsible for the reconstitution of T-cell immunity. Hence, Tα1 was first purified from TF5 in 1977 [34] and has been found to be 10–1000 times as active as TF5 evaluated *in vivo* and *in vitro* [47].

Tα1 is the asparaginyl endopeptidase cleavage product of prothymosin α (ProTα), an acidic nuclear protein consisting of 109 amino acid residues [10]. Tα1 is a highly conserved acid peptide, ubiquitously existing in lymphoid tissues such as spleen and lymph nodes, non-lymphoid tissues such as lungs, kidneys, and brain, but mainly existing in thymus gland [33], especially in the thymic epithelial cells. Interestingly, the secretion of Tα1 is not modulated by other hormones or releasing factors [54]. As a potent biological response modifier (BRM), Tα1 has intensive clinical applications. In the first randomized double-blind Phase II trial of Tα1 carried out by Schulof et al. [68], administration of synthetic Tα1 to postradiotherapy patients with non-small cell lung cancer exhibited significant improvements in relapse-free and overall survival, which was most pronounced in patients with nonbulky tumors. Now Tα1 is in clinical trials worldwide for the treatment of several types of cancer, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, which connect closely with hepatocellular carcinoma (HCC) [77]. Additionally, Tα1 shows remarkable effects in the treatment of other diseases such as severe sepsis [87,43], acute respiratory distress syndrome (ARDS) [38], severe acute respiratory syndrome (SARS) [23], gastrointestinal and systemic infectious disorders [39], and spontaneous peritonitis in individuals with cirrhosis [49].

Because of the extensive applications of Tα1, there is an increasing need to produce Tα1 in larger quantities to keep up with the growing clinical demand. Besides isolated from calf thymus, bioactive Tα1 can be obtained by solid-phase synthesis [78] or genetic engineering [80], but Tα1 currently used in clinic is entirely solid-phase synthesized polypeptide, with chemical features identical to the human Tα1 [40]. Recently, genetic engineering expression of Tα1 in different hosts including *Escherichia coli*, *Pichia pastries* and plants [55,14] has attracted more attention due to its potential for producing low cost and bioactive Tα1.

In this review, we briefly describe the biological activities of Tα1 and discuss the current applications of Tα1 in cancer and infectious diseases. Furthermore, we summarize ways of genetic engineering production of this peptide, which maybe provide a conceptual framework for future studies to improve the quality and the yield of Tα1 for different fields of research and clinical applications.

2. Biological activities of Tα1

2.1. Immunoregulation

Many studies have been performed to identify the immunoregulatory activity of Tα1 *in vitro* and *in vivo*. Evidence has shown that Tα1 increased the efficiency of T cell maturation [1], stimulated

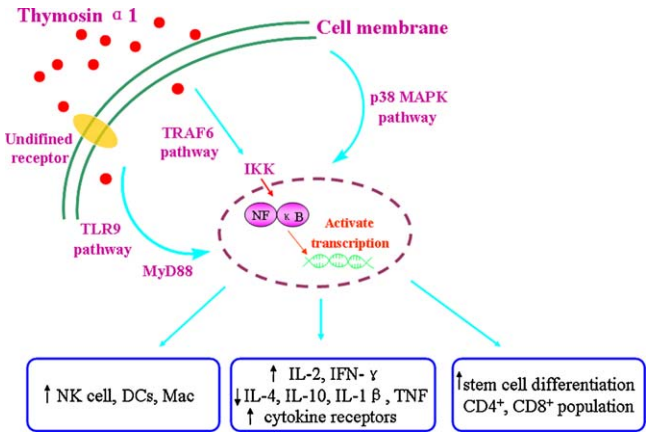

Fig. 1. Immunoregulation of Tα1 and action mechanisms. TRAF: TNF-receptor-associated factor; TLR9: toll-like receptor 9; Mac: macrophage; p38MAPK: p38 mitogen-activated protein kinase; IKK: I-kappa B kinase; MyD88: myeloid differentiation factor 88.

precursor stem cell differentiation into the $CD4^+/CD8^+$ T cells [57] and balanced $CD3/CD4^+/CD8^+$ T cells of peripheral blood mononuclear cells (PBMCs) [84]. By stimulating natural killer (NK) cells and cytotoxic lymphocytes ($CD8^+$ T cell), Tα1 could directly kill virally infected cells [67]. By activating dendritic cells (DCs), Tα1 was able to protect immunocompromised mice from death caused by aspergillosis [62]. Tα1 stimulated a significant increase of IL-2 and led to a decrease in the Th2 cytokines such as IL-4 and IL-10 in patients with chronic HCV [67]. Besides, Tα1 remarkably decreased the severity of severe acute pancreatitis by having a negative effect on serum levels of IL-1β and tumor necrosis factor-alpha (TNF-α) [84]. Tα1 also upregulated specific cytokine receptors such as high-affinity IL-2 cytokine receptors [42].

Not only activating immuno-effector cells or modulating cytokines expression, Tα1 also directly exerted its effects on target cells. It could increase the expression of MHC I [30] and tumor antigens [25], directly depress viral replication [4], and increase expression of viral antigens on the surface of target-infected cells [24], making them more visible to the immune system and less prone to escape from immunosurveillance.

Although the observations of the Tα1 potential immunoregulatory effects are clearly evident, what is not clear is the mechanisms of action on the immune system. It was reported that Tα1 could directly modulate the expression of cytokine genes, MHC class I, MHC class II related genes as well as a significant number of new genes, acting as immune system regulators [26]. Naylor and his colleagues demonstrated that genes of major histocompatability proteins, costimulatory molecules, chemokines and cytokines, and their receptors were upregulated in both T cells and monocytes exposed to Tα1 [54], indicating that there were multiple targets for its immune-enhancing activity. As illustrated in Fig. 1, Tα1-mediated stimulation of intracellular signaling pathways included mitogen-activated protein kinase (MAPK) transduction pathways [71] and TNF-α receptor-associated factor 6 (TRAF6) signal pathway by activating I-kappa B kinase (IKK) [88]. Tα1 has also been found to induce IL-6, IL-10 and IL-12 expression via IRAK4/1/TRAF6/PKCζ/IKK/NF-κB and TRAF6/MAPK/AP-1 pathways [56]. These pathways are shared by many cytokines, which predict potential synergy between Tα1 and cytokines. Tα1 was able to prime DC for antifungal Th1 resistance through Toll-like receptor 9 (TLR9)/myeloid differentiation factor 88 (MyD88)-dependent signaling [62]. Besides, DCs could also be primed by Tα1-induced activation of p38 MAPK, NF-κB pathways [83]. Activated plasmacytoid DCs (pDC) led to the activation of interferon regulatory factor 7 and the promotion of the IFN-α/IFN-γ-dependent effector path-

Table 1
Summary of antitumor activities of Tα1.

Author/reference	Object	Tumor model	Treatment	Dose/route/duration
Qin et al. [60]	HepG2 cells and SPC-A-1cells	Hepatocarcinoma lung and adenocarcinoma	Tα1	50 μg/mL
Beuth et al. [6]	BALB/c-mice	Liver and lung metastases	Tα1	0.01–10 μg/subcutaneous injection/7 days
Moody et al. [52]	Fisher rat	Mammary carcinogenesis	Tα1	10 μg/subcutaneous injection
Moody [53]	Fisher rat and C3(1)SV40T antigen mouse	Breast adenomas	Tα1	0.4 mg/kg/subcutaneous injection
Moody [53]	A/J mice	Lung adenomas	Tα1	0.4 mg/kg/subcutaneous injection/8 months
Chen et al. [12]	ICR mice	Hep-A-22 liver tumor	Plasmid–liposome complex containing Tα1 gene and IFNω_1 gene	40 μg plasmid DNA/tail vein injection/7 days
Garaci et al. [26]	BDIX rats	DHD-K12 colorectal cancer	5-Fluorouracil (FU) + IL-2 + Tα1	Not detailed
Sungarian et al. [73]	Long Evans rats	Glioblastoma	Carmustine (BCNU) + Tα1	45–200 μg/kg intraperitoneal injection/3 days

way, which resulted *in vivo* in protection against primary murine cytomegalovirus infection [63]. Moreover, activated indoleamine 2,3-dioxygenase (IDO) could induce transplantation tolerance and reduce inflammation allergy [64]. Recently, Qin et al. found that Tα1 inhibited HepG2 cells proliferation might associated with protein kinase B (Akt) signaling pathway [60].

2.2. Antitumor

Tα1 has been shown to decrease tumor cell growth both *in vitro* and *in vivo* and has been demonstrated therapeutic usefulness in several types of cancer (Table 1). Tα1 was observed to exhibit anti-proliferative effects on HepG2 human hepatoma cells and SPC-A-1 lung adenocarcinoma cells *in vitro* assays [60]. To explore the anti-metastatic/antitumor activity of Tα1, it was subcutaneously injected into BALB/c-mice, which significantly reduced liver and lung metastases and decreased local tumor growth [6]. Moody et al. investigated the effects of Tα1 on mammary carcinogenesis in fisher rats and found that Tα1 could reduce mammary carcinoma incidence and prolong survival time [52]. In another breast adenoma model, Tα1 increased the survival time in female C3(1)SV40T antigen transgenic mice and fisher rats, but it remained to be determined whether the immune response also increased or not [53]. The antitumor activity of Tα1 was most effective when the lung adenomas were small, which was based on studies proformed by Moody who gave Tα1 daily to A/J mice bearing lung adenoma [53]. Tα1 may fight against tumors through either stimulating the immune system or directly inhibiting the proliferation of tumor cells.

Tα1 in combination with other BRMs or chemotherapy agents also displays good effects in reducing tumor burden and progression. The plasmid–liposome complex containing the cDNA of human Tα1 and IFN ω1 was injected into ICR mice, and the dual-gene plasmid–liposome complex showed stronger inhibitory effect on the growth of tumor than the single gene of Tα1 or IFN ω1, which might attribute to indirect and additive induction of apoptosis of tumor cells by the increased expression of Tα1 and IFN ω1 [12]. In DHD-K12 colorectal cancer model, combination of 5-FU, IL-2 and Tα1 could dramatically increase survival rates as well as control tumor metastasis [26]. Similarly, compared with Carmustine (BCNU) monotherapy, intraperitoneal injection of Tα1 and BCNU to adult Long Evans rats bearing glioblastoma could significantly lower the tumor burdens and increase the cure rates [73]. Since the cascades and feedback networks of immune responses, the combination of immunoactive molecules that affected different immune effector cells resulted in a stimulation of the immune response significantly stronger than that evoked by single treatments. This could contribute in helping explain the mechanisms of the significance of combination therapy.

2.3. Protection against oxidative damage

Several reports showed that Tα1 had protective effects against oxidative damage. Tα1 had a positive influence on liver superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity and thereby limited free radical damages to hepatic tissue [5]. Similarly, it was reported that Tα1 could ameliorate streptozotocin-induced pancreatic lesions and diabetes by reducing malondialdehyde (MDA), increasing GSH level and enhancing the activities of both SOD and catalase (CAT), suggesting that Tα1 treatment could greatly enhance the overall antioxidative capability of pancreatic tissues [61].

2.4. Other functions

Tα1 possesses the ability of influencing the central nervous system [68,70]. Its modulatory effect on the excitatory synaptic transmission in cultured hippocampal neurons was documented [81]. Similarly, when it was combined with chemotherapeutics in treating cancers, Tα1 could prevent patients from chemotherapy-induced neurotoxicities [2]. Moreover, Tα1 has potent effects in promoting endothelial cell migration, angiogenesis as well as wound healing [50].

3. Clinical application of Tα1 in treating cancers and infectious diseases

3.1. Applications of Tα1 in cancers

Patients with cancer are often accompanied with significant deficiencies in cellular immunity. In addition, standard treatments for cancer usually induce significant depression of the immune response. Tα1 has been demonstrated to decrease tumor cell growth both *in vitro* and *in vivo* and has therapeutic effect in several types of cancer. In advanced lung or advanced breast cancer, Tα1 combined with chemotherapy could prevent patients from chemotherapy-induced neurotoxicities [2]. In a Phase II multicenter, randomized open-label study, different dose levels of Tα1 in combination with Dacarbazine (DTIC) chemotherapy were given

to patients with stage IV melanoma. Reported results show that the combination therapy tripled the overall response rate and extended overall survival by nearly 3 months compared with patients treated with DTIC, combined with IFN-α [8]. More recently, in patients with unresectable HCC, transarterial chemoembolization (TACE) combined with Tα1 resulted in numerically higher rates of survival and tumor response, including transplant candidacy, with fewer bacterial infections, than TACE alone [29].

Obviously, significant tumor growth inhibition and survival rate increase were achieved in different human tumor models when Tα1 was combined with other treatment modalities. It can be concluded that combinatorial therapies, in which Tα1 represents one important mediator, are effective therapeutic strategy against tumors and will be the key focus for the use of Tα1 in treating cancers in the future.

3.2. Hepatitis B

Chronic HBV infection is a serious clinical problem because of its worldwide distribution and potential adverse sequelae, such as cirrhosis and hepatocellular carcinoma [44]. Tα1 has been approved for the treatment of hepatitis B in many countries worldwide with a significantly increasing virological response over time after therapy [58]. Most of the studies have evaluated the efficacy of Tα1 in the treatment of HBeAg-positive and HBeAg-negative chronic hepatitis B. For instance, administering Tα1 either 0.8 mg or 1.6 mg to 316 Japanese patients with HBeAg-positive chronic hepatitis B showed HBeAg seroconversion in 18.8% and 21.5% at 48 weeks after the end of treatment, respectively [37]. Similarly, administering Tα1 1.6 mg to Chinese patients with HBeAg-negative chronic hepatitis B twice weekly showed a complete response, defined as normalization of alanine transaminase (ALT) and undetectable HBV DNA by PCR assay, in 11 of 26 patients (42.3%) at 6 months after the end of treatment [86]. Zhang et al. searched materials from different databases and analyzed eight trials using meta analysis. They found that lamivudine and Tα1 combination treatment was particularly prominent than lamivudine monotherapy in terms of ALT normalization rate, virological response rate and HBeAg seroconversion rate [89]. Conversely, Lee et al. [41] revealed that combining Tα1 and lamivudine did not display a better benefit to virological and biochemical response than the lamivudine monotherapy. Maybe the small trial scale led to the divergent results.

3.3. Hepatitis C

As a monotherapy, Tα1 does not seem useful in treating HCV infection, which is confirmed by a randomized, double-blind, placebo-controlled trial [3]. However, combination therapy of Tα1 and pegylated interferon α2a (peg-IFN-α2a) could effectively suppress viral replication in difficult-to-treat hepatitis C patients. In addition, Tα1 was well tolerated with no significant adverse effects observed [66]. Approximately 50% of treatment-naive HCV patients failed to achieve a sustained virologic response (SVP) with standard peg-IFN and ribavirin therapy [21], so a triple combination therapy with peg-IFN-α2a, ribavirin and Tα1 has been developed and proved to be a safe [7] and effective [59] treatment option for difficult-to-treat HCV patients who are refractory to prior conventional treatment.

3.4. AIDS

Human immunodeficiency virus (HIV) specially targets cells that express CD4, such as macrophages, DCs and $CD4^+$ T cells. When the virus becomes lymphotropic, it begins to infect $CD4^+$ T cells efficiently followed by significantly declined antibody class switching. Furthermore, $CD8^+$ T cells are not stimulated as effectively, facilitating the escape of the virus from immune control and the collapse of the whole immune system [51]. Since significant immune responses play an important role in the prevention of infection with human HIV, it is thought that the induction of strong immune responses especially CTL responses against HIV-1 could be important to prevent the onset of acquired immune deficiency syndrome (AIDS) [74]. One study has suggested that combination of Tα1, zidovudine (AZT) and IFN-α resulted in a significant increase in the number and function of $CD4^+$ T cells and a reduction in HIV titers [27]. Another interesting finding was provided by Chadwick et al. [9], who studied the safety and efficacy of Tα1 in combination with highly active antiretroviral therapy (HAART) in stimulating immune reconstitution. The results demonstrated that Tα1 appeared to be very well tolerated and could dramatically increase the levels of signal joint T cell receptor excision circles (sjTREC) in patients with advanced HIV disease. However, longer treatment duration of Tα1 in augmenting the immune reconstitution needs further investigation.

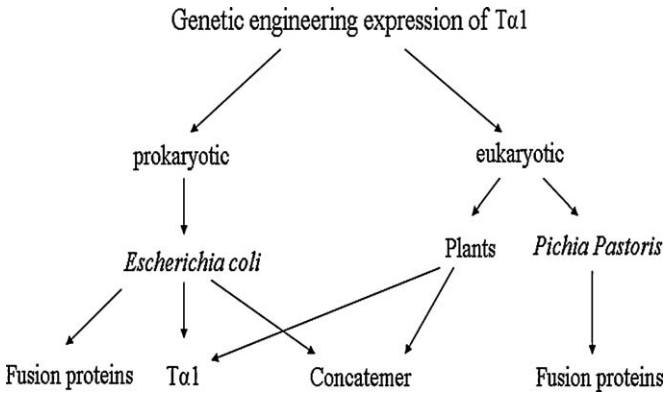

Fig. 2. Ways to genetic engineering expression of Tα1. Production of Tα1 in prokaryotic and eukaryotic expression systems is a cost-effective approach. Tα1, Tα1-related fusion proteins and Tα1 concatemer can be expressed in *E. coli*. Tα1 as well as several Tα1-related fusion proteins are expressed in *P. pastoris*. Besides, plants are also used for the production of Tα1 in the form of monomer or concatemer.

4. Gene expression of Tα1

From the natural source, Tα1 can only be obtained in tiny quantities. To obtain larger amounts of this peptide, literally tons of fresh frozen calf thymus tissue and acetone are required [31]. Furthermore, heterogeneous allergens introduced by manufacturing process limit its availability for research and medical applications. Solid phase synthesis has permitted scientists to synthesize and purify Tα1 to allow human clinical trials [78,79] with the advantages of simplicity, ease of operation, general efficiency and lack of endotoxins and DNA contaminations. However, difficult sequences Tα1 bears and the high number of protecting groups required to assemble the peptide may give final low yields, insufficient purity and high expenses. A recent report revealed that combination of the side-chain anchoring approach with the hydrophilicity of the totally PEG-based resin facilitated the synthesis of Tα1 in high purity and high yields [28].

With the advancement in genetic engineering, bioactive Tα1 can be expressed in prokaryotic and eukaryotic expression systems, which are cost-effective alternative approaches to produce biotechnical drugs. These products including Tα1, fusion proteins and concatemers produced in *E. coli*, *P. pastoris* and plants were all soluble expressed, which could escape from refolding from inclu-

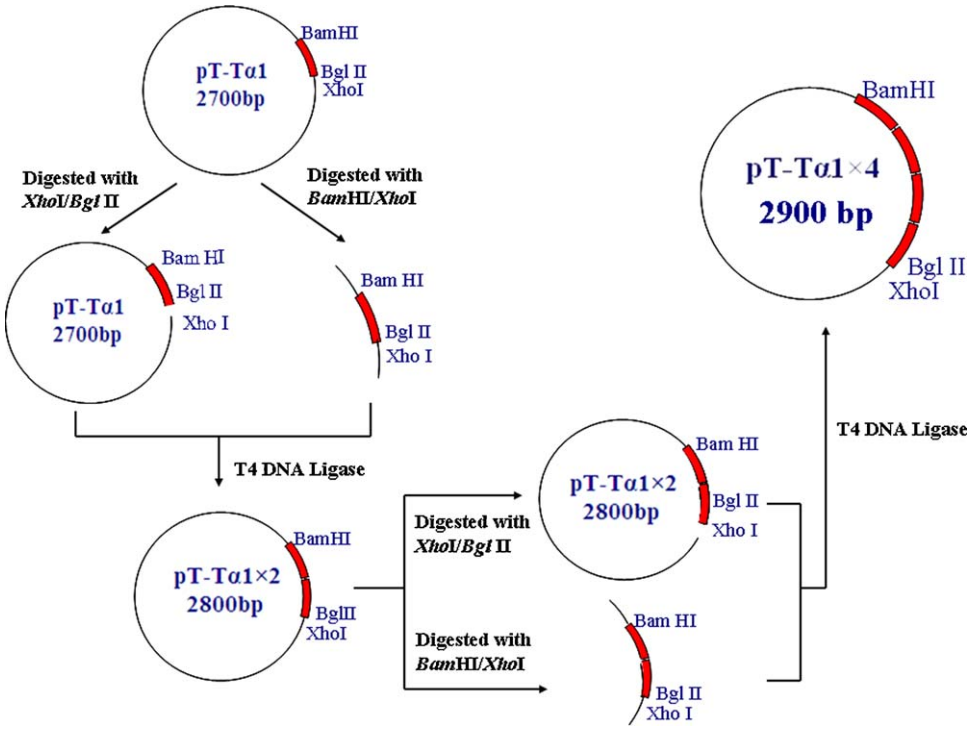

Fig. 3. Construction of the concatemer gene. A T-vector containing the Tα1 gene (in red) was digested with BamHI/XhoI and BglII/XhoI respectively. When digested with BglII and BamHI, the two fragments had identical termini and could be ligated with T4 DNA ligase subsequently forming a new sequence GGATCT, which could not be digested by neither BamHI nor BglII. Therefore, a plasmid containing double Tα1 genes was constructed and could not be destroyed when the concatemer *Tα1* gene of 4 repeats was constructed. Thus, the plasmid containing concatemer *Tα1* gene of 4 repeats and 6 repetats could also be constructed [90]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sion bodies (see Fig. 2). But isolation and purification of them with a high purity is difficult and applying them to clinical treatment has not come true. A number of studies of using genetic engineering to gain bioactive Tα1 are summarized as follows.

4.1. Prokaryotic expression of Tα1

E. coli is one of the earliest and most widely used hosts for the production of heterologous proteins [75]. It has the advantages of rapid growth and expression, easy culture and high yields. However, *E. coli* is not the system of choice for expressing disulfide rich proteins and proteins that require post-translational modifications [17]. With the characteristics of small molecular weight and needless post-translational modifications, Tα1 is suitable to be expressed by *E. coli* system. Several strategies for the expression of Tα1, Tα1-BRMs fusion proteins, Tα1 concatemer and so on using *E. coli* expression system have been reported.

4.1.1. Expression of Tα1

Generally, small peptides are difficult to be overexpressed directly in *E. coli* since they can be quickly degraded by cellular proteases. The use of protease-deficient host strains and fusion tags, such as his-tag and thioredoxin can help to avoid non-specific proteolytic degradation and facilitate purification. Following this approach, the synthesized human Tα1 gene was inserted into pET-28a (+) plasmid and then inductively expressed as a soluble form in *E. coli* BL21, which is a protease-deficient host strain. Compared with other expression systems, the BL21/pET-28a system provided the highest expression level of fusion protein, which amounted to 70% of total expressed proteins [13]. Furthermore, Tα1 gene was inserted into pET32b (+) and expressed with thioredoxin in *E. coli* strain ER2566. After proteolytic cleavage and chemical acetylation, the resultant Tα1 was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) with the yield of 29 mg per litre of bacterial culture. This method is simple, cost-effective and suitable for large-scale production of Tα1 [18].

4.1.2. Expression of Tα1-BRMs fusion proteins

Since improved control of tumor growth can be observed when tumor-bearing mice were treated with Tα1 and high doses of IL-2 [46], combination therapies have performed and have been proved to be effective in inhibiting tumor growth and in controlling infectious diseases especially in the immunocompromised host. Thus, the expression of fused molecules of Tα1 and other BRMs which have synergistic effect with Tα1 was investigated. Tα1 and cBLyS, a soluble B-cell lymphocyte stimulator amplifying the humoral response, were fused with a flexible linker sequence and expressed in *E. coli*. This bifunctional lymphokine was useful in the treatment of various immunodeficiency syndromes and served as an immunomodulator to enhance the host's response to vaccination [69]. The fusion protein of Tα1 and consensus IFNα (IFNα-con), which was soluble and amounted to more than 20% of total proteins of *E. coli*, showed higher antiviral effect than IFNα and the activity in promoting lymphocyte proliferation was similar to commercial Tα1 [45].

4.1.3. Expression of Tα1 concatemer

It is difficult to extract and purify Tα1 from the fermentation broth since its molecular weight is small. The concatemer strategy maybe partially solve the problem of low expression and the difficulty of purification by increasing the size of the target molecule. A concatemer *Tα1* gene of 6 repeats was constructed according to the *E. coli* codon usage preference, ligated with expression vector pET-22b (+) and transformed into *E. coli* BL21 (DE3) [90]. The Tα1 monomer was successfully released by hydroxylamine inci-

sion after concatemer purification, and its activity in promoting mice splenic lymphocyte proliferation was approximatively identical to the natural Tα1. The intimate process of construction of the concatemer *Tα1* gene is described in Fig. 3.

In addition, a concatemer *Tα1* gene of 4 repeats was synthesized and successfully expressed in *E. coli* in a soluble form. Preliminary results demonstrated that the concatemer protein also had the activity in stimulating mouse spleen lymphocyte proliferation [15].

4.1.4. Co-expression of Tα1 and RimJ

It is a common knowledge that *E. coli* lacks efficient post-translational modification systems for modifying exogenous proteins. However, Fang et al. [20] found that the fusion protein of Tα1 and ribosomal protein L12 was partly N^{α}-acetylated when expressed in *E. coli* and this modification was performed by RimJ, which is the N terminal acetyltransferase that modifies the ribosomal protein S5 [85] and acts as an ribosome assembly factor [65]. This enlightens us that fully acetylated Tα1 can be obtained by co-expressing with RimJ. However, little is known about the pathway by which this fusion protein is N^{α}-acetylated. The previous reports that the activity of none or partly N^{α}-acetylated Tα1 is similar to the natural one [80] illustrated that N^{α}-acetylation of Tα1 could influence the stability of the peptide instead of the bioactivity *in vivo*.

Based on the above research findings, it can be concluded that Tα1 is suitable to be expressed by *E. coli* expression system. To improve the expression efficacy, the following measures may be meaningful: (i) choosing *E. coli* usage preference codons; (ii) using different promoters to regulate expression; (iii) using protease-deficient host strains.

4.2. Eukaryotic expression of Tα1

Yeasts are attractive hosts for the production of heterologous proteins for providing post-translational modifications and generating stable cell lines via homologous recombination [16]. Some examples of expressing Tα1 in yeast expression system are presented as follows.

Chen et al. [11] successfully constructed an effective yeast expression system for Tα1 in which pYES2-Tα1 plasmid was transformed into INVSc1 yeast host strain, and Tα1 expressed by this system could improve the level of $CD8^{+}$ cells in BALB/c mice treated with cyclophosphamide in advance.

Fusion expression of Tα1 and other BMRs in *P. pastoris* are also reported. IFNα2b exhibits synergic effects with Tα1 in the treatment of hepatitis B and hepatitis C. The fusion protein of IFNα2b and Tα1 linked by different lengths of (Gly–Gly–Gly–Gly–Ser)*n* (*n* = 1–3) were expressed in *P. pastoris* and exhibited both antiviral activity of IFNα2b and immunomodulatory activity of Tα1 estimated *in vitro* [82]. Thymopentin (TP5) not only acts as an immunomodulatory factor in cancer chemotherapy, but is also a potential chemotherapeutic agent in the human leukemia therapy [19]. However, extremely short half-life *in vivo* (30 s) [76] greatly restricts its clinical applications. In this sense, a Tα1–TP5 fusion gene was synthesized, inserted into vector pGAPZαA and expressed in *P. pastries* by our research team [22]. The Tα1–TP5 fusion peptide displayed higher activity than Tα1 and TP5 in promoting the phagocytosis of macrophages and the proliferation of Kunming mouse splenocytes.

Plants are also used for the production of Tα1 in the form of monomer or concatemer [55,14]. Recently, the concatemer *Tα1* gene of 4 repeats was introduced and successfully expressed in transgenic tomatoes. The bioactivity of concatemer protein for stimulating proliferation of mice splenic lymphocytes *in vitro* was stronger than that synthesized artificially or Tα1 concatemer protein expressed in the *E. coli* system, but the underlying reasons were unclear and required further investigation [14].

These examples demonstrate that bioactive Tα1 can be obtained by genetic engineering. With great efforts are being made, such as improving the quality, functionality, purity and yield of Tα1 products, it can be expected that over the next few years they will find their way into the clinic.

5. Conclusions

In addition to immunomodulatory activity, Tα1 owns the ability of influencing the central nervous system and regulating endocrine system. It is very likely that due to its pleiotropic biological activities, Tα1, either alone or combined with other treatment strategies, will have a broader spectrum of applications for successful treatment of various diseases in clinic. Up to now, chemical synthesis is the only effective way to produce Tα1 for clinical therapy. Genetic engineering is an attractive alternative route of expressing bioactive Tα1, but at present, it offers no higher purity of Tα1 compared with chemical synthesis. Recently, gene expression of Tα1 concatemer and fusion proteins have become a major research focus, which are effective strategies for facilitating purification, increasing production and reducing production costs. It is believed that the rapid development of biotechnology may allow application of Tα1 products obtained by genetic engineering in clinic in the future.

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