Original Article

Effect of Tianshengyuan-1 (TSY-1) on telomerase activity and hematopoietic recovery - in vitro, ex vivo, and in vivo studies

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Abstract: Aplastic anemia is a heterogeneous disorder of bone marrow failure syndrome. Accumulating evidence indicates that both acquired and congenital aplastic anemia is linked to telomerase activity and telomere length. Chinese herbal medicine Tianshengyuan-1 (TSY-1), a liquid extraction of multiple Chinese herbs, appears to stimulate hematopoiesis in patients with bone marrow deficiencies; however, the exact mechanism of action remains unclear. In this study, we investigated the effect of TSY-1 on telomere length and telomerase activity. We first investigated the effects of TSY on cultured cell lines including CD34+ hepatic stem cells and CD4+/CD8- Jurkat cells. An immune-mediated murine aplastic anemia model and human samples, including peripheral blood samples of 4 healthy donors and bone marrow hematopoietic cells from 4 patients with hypocellular myelodysplastic syndrome (MDS), were also used to test the efficacy of TSY on hematopoiesis, telomerase activity and telomere length. Our results indicated that TSY-1 increased the telomerase activity and telomere length in a dose-response manner in vitro, in vivo, and in human samples including 3 of 4 healthy individuals and 3 of 4 bone marrow samples from MDS patients. In immune-mediated murine aplastic anemia model, TSY-1 activity on Telomere length was parallel to the significant increasing of the RBC, hemoglobin, hematocrit, and platelet count in peripheral blood, increasing of CD34+ cell count and hematopoiesis, and decreasing of fatty infiltration in bone marrow samples. Our study demonstrated that TSY-1 may exert its effects by modulating telomerase activity of hematopoietic cells. Further studies are warranted to explore the precise molecular mechanisms of how TSY-1 regulates telomerase activity and telomere length, and also to test the TSY-1 in randomized control trials.

Keywords: TSY-1, hematopoietic cells, telomerase, telomere

Introduction

Numerous acquired or congenital conditions can lead to bone marrow deficiency, of which the most severe form is aplastic anemia (AA). AA is a life-threatening disease that causes bone marrow failure with markedly hypocellular marrow and low peripheral blood cell counts [1]. Its high mortality rates are partly attributed to the lack of effective treatment methods [2]. Bone marrow transplant with or without immune suppression is currently the treatment modality for this condition, but the overall effectiveness of this method is limited [3, 4]. While the exact mechanism of the disease remains to be determined, recent studies have linked the disease to the dysfunction of telomerase enzyme activity and telomere length [5-7]. About one third of patients with AA, especially those who do not respond to immunosuppressive therapy, have shortened telomeres in leukocytes [8, 9]. Some of the shortened telomeres are associated with specific mutations identified in telomerase genes [10].
Chinese herbal medicine Tianshengyuan-1 (TSY-1), a liquid extract of multiple Chinese herbs, appears to have effects in stimulating hematopoiesis in patients with bone marrow deficiencies including AA [11-15]. The exact mechanism of action remains unclear. Recently, a study that utilized mice with chemically-induced bone marrow failure highlighted the efficacy of the drug in inducing bone marrow hematopoiesis [12, 13]. Mice treated with combined cytotoxic agents of cyclophosphamide (50 mg/kg) and benzidine (0.5 ml/kg) resulted in complete bone marrow destruction. Compared to untreated control mice, mice treated with TSY-1 showed significant recovery of hematopoietic cells in both bone marrow and peripheral blood in a dose-response manner [12, 13]. Interestingly, TSY-1 induces extramedullary hematopoiesis in multiple organ systems including both liver and spleen, and also specifically increases the number of CD4+/CD8- T cells and CD34 cells [12], all of which are commonly seen to be reduced in AA patients [16-18].

The purpose of the current study was to study the potential molecular mechanisms of TSY-1 on bone marrow deficiency, focusing on telomere length and telomerase activity. We first investigated the effects of TSY on telomerase activity and telomere length on a number of in vitro cultured cells including CD34+ hepatic stem cells and CD4+/CD8- Jurkat cells. To test the efficacy of TSY-1 on hematopoiesis and telomerase activity in vivo, an immune-mediated murine AA model established with high dose radiation followed by allogeneic infusion of thymic cells was utilized. Furthermore, TSY-1 has proven effects on telomere length and telomerase activity in human samples including enriched T lymphocytes obtained from peripheral blood samples of four healthy donors. Since AA patients are rare and patient samples are difficult to obtain, we analyzed bone marrow samples from four patients diagnosed with hypocellular myelodysplastic syndrome (MDS) who had refractory anemia. Hypocellular MDS has overlapping clinical and pathological features to AA, and is also associated with abnormality in telomerase activity [9, 10].

Materials and methods

Reagents

TSY-1 liquid, 10 ml/amp, batch No. 06121601, content of crude drug of 0.31 g/ml, was provided by Beijing Tianyuyiming Biological Technology Co., Ltd. Non-transformed CD34+ human liver stem cells and CD4+/CD8- Jurkat cells were kindly provided by Dr. Genhong Cheng’s lab at University of California, Los Angeles.

Cell culture and TSY-1 treatment

CD34+ liver stem cells were cultured in RPMI-1640 Medium supplement with fetal bovine serum to a final concentration of 20% at 37.0°C temperature and 5% CO₂. Jurkat cells (CRL-2679™, ATCC Co. CD8-lymphocyte) were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.4 mg/ml G418, and 10% fetal bovine serum. The cells were also maintained at 37.0°C with 5% CO₂. For both cell lines, medium was replaced every 2 to 3 days depending on cell density, which was maintained around 2 × 10⁵ viable cells/ml before TSY-1 treatment. The cultured cells were treated with 1/16, 1/8, and 1/4 dilutions of TSY-1 stock solution that were equivalent to concentrations of 20 µg/ml, 40 µg/ml, and 80 µg/ml respectively, for 24 hours prior to harvesting for the analysis of telomere length and telomerase activity. As a control, cells cultured in medium without treatment of TSY were also harvested for analysis.

Telomerase activity assay

To determine the telomerase activity, harvested cells (about 1 × 10⁶) were first washed with PBS, followed by RNA extraction, which was used to determine the enzymatic activity. Enzymatic activity was detected by the PCR-based telomeric repeat amplification protocol (TRAP), using the Quantitative Telomerase Detection Kit and following the manufacturer’s instructions (US Biomax, Catalog No. MT3010). Each assay mixture included 12.5 µl of 2 × QTD Premix, 1.0 µl cell extract and 11.5 µl PCR qualified water. A dilution series of TSR control template was prepared in lysis buffer to serve as a standard curve. Reactions were set up in triplicates.

The quantity of telomerase substrate produced in each well from the telomerase activity of 1 µl of cell extract within 20 min was determined from a linear plot of the log₁₀ of the quantities of TSR control template standards versus the
Ct values for their wells. Telomerase activity was determined as the relative ratios of the mean quantity of telomerase substrate in each TSY treated sample to the mean quantity of telomerase substrate in the untreated control sample.

**Telomere length assay**

To determine telomere length, genomic DNA was extracted from the above treated cells and telomere length was determined using methods described by Gu [19]. The telomere was amplified with primers 5’-CGG TTT GTT GGG TGG TGG TGG TGG GCT TAC CCT TAC CCT TAC CCT TAC CCT-3’ and 5’-GGC TTG CCT TAC ACT AGC-3’ and 5’-CAC CAA CTT CAT CCA GTT TCA CC-3’. As the internal standard, Hgb was amplified with primers 5’-GCT CTA ACG ACA ACT GTG TTC ACT AGC-3’ and 5’-CAT CCA GTT TCA CC-3’. The reaction mixture was subjected to 1 PCR cycle at 50°C for 2 minutes, 95°C for 10 minutes and 40 PCR cycles at 95°C for 15 seconds, 56°C for 1 minute (with Tel primer) or at 58°C for 1 minute (with Hgb primer).

Quantitative analysis was performed by the software Gene Expression Analysis of the iCycler iQ Real-Time PCR Detection System. The standard curve for linearity was evaluated, and the acceptable \( R^2 \) was ≥0.98. The Ct values for all samples, including the calculated mean Ct and standard deviation, were calculated. The experiment was repeated for samples with standard deviation >0.3. The quantity for each sample was determined through dividing the mean Ct from the telomere length assay (that used Tel primers) by the mean Ct from the internal control assay (Hgb primers). Telomere length was determined as the relative ratio of the mean quantity of each sample (TSY treated cells) to the mean quantity of sample control (TSY untreated cells).

**Establishment of immune-mediated AA model**

DBA/2 mice and BALB/c mice were raised and housed in specific pathogen-free (SPF) conditions at the Institute of Laboratory Animal Science, CAMS & PUMC in China. All mice were male, with a weight range of 20 g to 22 g. To prepare for thymic suspension after sacrificing the DBA/2 mice, thymus were harvested and gently homogenized in phosphate-buffered saline (PBS) containing 10% fetal bovine serum, filtered through a 200 mesh stainless filter. Thymic cells were then washed in phosphate-buffered saline, stained with Trypan Blue for viability and counted using light microscopy. Cells were then prepared into single cell suspension in RPMI 1640 (5 × 10^5/mL) and stored in the 37°C incubator till use.

BALB/c mice were divided into five groups: control (n=21), model (n=33), TSY-1 low dosage (n=24), TSY-1 medium dosage (n=24), and TSY-1 high dosage (n=24), altogether a total of 126 mice were studied. The mice in the model and three TSY-1 groups received 4.0 Gy doses of 60Co γ ray exposure at a rate of 1.0 Gy/min × 5 min followed by injection of thymic cell suspension with a dose of 0.2 ml/mouse through the lateral tail vein (1 × 10^6 cells/mouse). The control group mice received pseudo-irradiation but also received the same dose of thymic cell suspension injection as the other groups of mice. The control and model groups of mice were administered 0.4 ml of distilled water intragastrically twice per day. The TSY-1 low, medium, and high dosage groups of mice were administered 0.15 ml (=3.2 g/kg), 0.3 ml (=6.4/ kg), and 0.6 ml, respectively (=12.8 g/kg). The total treatment period was two weeks (14 days).

**Peripheral blood count and telomere length analysis**

A 0.2 ml of blood sample was collected from the eye socket of each mouse 30 minutes after the last dosage of treatment and was stored in a Heparin anti-coagulated vial. The sample was divided into 2 parts: 0.1 ml for routine blood count and 0.1 ml for telomere length analysis. For the former sample, red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin concentration, reticulocyte count, reticulocyte percentage and hematocrit were measured and calculated using standard laboratory techniques. For telomere length analysis, the sample was frozen at -80°C first and brought back to the laboratory at UCLA. Genomic DNA was extracted from the frozen blood sample and was used for telomere length analysis using the method as described above.

**Bone marrow analysis for CD34 by flow cytometry and histopathological analysis**

All BALB/c mice were sacrificed with cervical dislocation before the isolation of right femurs.
The femurs were divided equally into two parts: one part for flow cytometry analysis of CD34 positive cells and one part for histopathological analysis.

For flow cytometry analysis, bone marrow was flushed out by 800 ul of fetal bovine serum. Bone marrow cells were washed in PBS and vortexed after centrifugation. 500 ul of the bone marrow mixture was transferred to a tube and incubated for 30 minutes at 37°C in the dark with anti-mouse CD34-FITC. The acquisition was then carried out using FACS Calibur (BD Biosciences, USA) and data analyzed with CellQuest Software.

For histopathological analysis, femur bone fragments were fixed in 10% formaldehyde solution. Then, the tissue was transferred to 5% nitric acid solution for decalcification for 4 days. The decalcified tissues were then embedded with paraffin, and 4 µm thickness sections were prepared for H&E staining.

**Isolation human T lymphocytes from healthy human donors for TSY-1 treatment**

Peripheral blood samples were obtained from 4 healthy donors at the UCLA virology core following an institutional research board (IRB)-approved protocol. Normal human T lymphocytes were isolated from these samples according to the manufacturer's protocol (Ficoll-Paque PLUS, GE Healthcare). Isolated T cells were cultured in RPMI-1640 supplemented with 10% human serum and were exposed to TSY-1 (0, 20 and 40 µg/ml) for 24 hours. Cells were then harvested for the analysis of telomerase activity and telomere length using above described methods.
**Table 1.** Hematological results from all groups after 14 days of treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (10^9/L)</th>
<th>RBC (10^12/L)</th>
<th>HBG (g/L)</th>
<th>HCT (L/L)</th>
<th>PLT (10^9/L)</th>
<th>RET%</th>
<th>RET (10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (n=21)</td>
<td>8.48±2.83</td>
<td>10.78±0.27</td>
<td>170.29±4.68</td>
<td>0.47±0.01</td>
<td>728.1±135.91</td>
<td>2.72±0.5</td>
<td>0.59±0.11</td>
</tr>
<tr>
<td>Model control (n=33)</td>
<td>2.63±1.80***</td>
<td>8.46±0.70***</td>
<td>137.7±10.30***</td>
<td>0.38±0.03***</td>
<td>558.39±149.70***</td>
<td>3.61±1.610</td>
<td>0.6±0.23</td>
</tr>
<tr>
<td>TSY (3.2 g/kg) (n=24)</td>
<td>2.55±1.40</td>
<td>8.95±0.75*</td>
<td>143.41±14.59</td>
<td>0.4±0.03*</td>
<td>539.18±123.64</td>
<td>2.73±0.73</td>
<td>0.49±0.13</td>
</tr>
<tr>
<td>TSY-med (5.4 g/kg) (n=24)</td>
<td>2.59±1.69</td>
<td>8.97±0.48**</td>
<td>144.48±17.93*</td>
<td>0.4±0.02**</td>
<td>567.39±136.04</td>
<td>3.0±1.06</td>
<td>0.54±0.19</td>
</tr>
<tr>
<td>TSY-high (12.8 g/kg) (n=24)</td>
<td>2.52±1.46</td>
<td>9.14±0.77**</td>
<td>146.7±10.23*</td>
<td>0.4±0.03**</td>
<td>664.05±254.69**</td>
<td>3.23±0.61</td>
<td>0.59±0.11</td>
</tr>
</tbody>
</table>

*p<0.05; ***p<0.001, compared with normal control by Student t-test.

Isolation bone marrow hematopoietic cells from hypocellular MDS patients for TSY-1 treatment

With the IRB approval of UCLA, bone marrow samples that were remnants of conventional hematopathological analysis were obtained from 4 patients from the flow cytometry lab at UCLA, Department of Pathology and Laboratory Medicine. All four patients had established diagnosis of hypocellular MDS who had refractory anemia based on the WHO classification. Patient identification including name, hospital ID number, specimen number, and other relevant information were removed prior to the submission of samples to the laboratory for analysis. Hematopoietic cells were isolated according to the manufacturer’s protocol (Ficoll-Paque PLUS, GE Healthcare). Isolated hematopoietic cells were cultured in RPMI-1640 supplemented with 10% FBS and were exposed to TSY-1 (0, 1/8, 1/4) for 24 hours. Cells were then harvested for the analysis of telomerase activity using above described method.

Statistical analysis

Descriptive statistics, such as mean and standard deviation, were used to summarize the results. The Student’s t-test ANOVA were used for analysis of continuing variables. Statistical significance was defined by a two-tailed p-value of 0.05.

Results

**TSY-1 stimulated telomerase activity, increased telomere length, and cell proliferation in non-transformed CD34+ stem cells and CD4+/CD8- Jurkat cells**

As most of the commercially available CD34+ hematopoietic cells were derived from malignant leukemia patients, we tested the TSY-1 on a non-malignant CD34+ hepatic stem cell line instead. In addition, because the AA was associated with abnormal T-cell function, the effect of TSY-1 was also examined in the CD4+/CD8-negative Jurkat cells. As shown in Figure 1, TSY-1 increased the telomerase activity and telomere length in a dose-response manner in both cell lines. There was also an increased cell count in response to the treatment in both cell lines.

**Effects of TSY-1 in immune-mediated AA mouse model**

We then tested the effect of TSY-1 in the immune-mediated AA model. In this model, BALB/c mice exposed to 4.0 Gy Co60 γ followed by immune suppression with injection of thymic suspension obtained from DBA-2 mice effectively induced bone marrow deficiency as evidenced by the decrease of peripheral blood RBC count, WBC count, hemoglobin concentration, hematocrit, platelets count, and a slight increase of reticulocyte percentage in the model group relative to the untreated control group (**Table 1**). After 14 days of treatment, TSY-1 showed a significant dose-response increase of RBC, hemoglobin, hematocrit, and platelet count (**Table 1**). However, the WBC count and reticulocyte count did not change significantly.

**Figure 2** shows bone marrow CD34+ cells analyzed by flow cytometry and bone marrow histology in peripheral blood samples. Telomere length is also shown. A dose-response increase of CD34+ cells count was observed in response to TSY-1 treatment (**Figure 2A** and **2C**). Histological analysis showed that a marked decrease of hematopoietic elements as well as fatty infiltration were seen in model bone marrow sample relative to the untreated normal, confirming the effect of bone marrow deficiency in the model group. In TSY-1 treated groups, a dose-response increase of hematopoietic elements and a decrease in fatty infiltration were
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Figure 2. The effect of TSY on bone marrow CD34+ cells count, bone marrow histology and telomere length in immune mediate AA mouse model. A & C: Bone marrow CD34+ cell population analyzed by flow cytometry. Note significant increase of CD34 positive (**P<0.01 and ***P<0.001 by Student t-test) in TSY-1 treated group relative to the model control group. B: Histopathological study of bone marrow after treatment with TSY. D: Telomere length in peripheral blood sample, plotted the ratio of each TSY treated sample relative to the untreated control. The differences of telomere length among control and various dosage groups reached significance level by ANOVA test (P<0.05).

seen (Figure 2B). As shown in Figure 2D, a clear increase of telomere length in peripheral blood was evident in response to TSY-1 treatment.

Effects of TSY-1 in T-cells isolated from peripheral blood of healthy donor and bone marrow hematopoietic cells from patients with MDS

To further examine the effect of TSY-1 in human samples, peripheral blood from 4 healthy donors were analyzed after ex vivo culture of the cells in the presence of TSY-1. Figure 3 shows the effect of TSY-1 on telomerase activity and telomere length in normal T-cells isolated from four healthy donor peripheral bloods. The isolated peripheral blood T-cells were incubated with control, 20, and 40 µg/ml of TSY-1 for 24 hours. Three of four samples showed dose-response effects of TSY-1 on both telomerase activity and telomere length. Taking average of the four samples, the overall difference of these samples comparing treated groups versus control reached statistical significance even with the small sample size (P<0.05 by ANOVA test).

Due to the difficulty of obtaining samples from AA patients and the fact that there exists a significant clinicopathologic overlap between AA and MDS with refractory anemia, we tested the effect of TSY-1 in MDS patient samples instead. MDS represents a heterogeneous group of hematological conditions caused by ineffective hematopoiesis. Patients with MDS may have hypercellular, normocellular, or hypocellular marrows. Hypocellular MDS can produce similar pathologic findings as AA. Originally cultured hematopoietic cells were explanted from four hypocellular MDS patient bone marrow samples and cultured with TSY at the concentration of 0, 1/8, 1/4. Using real-time PCR, we found TSY increased telomere length of hematopoietic cells from MDS patients in a dose-response manner for 3 of 4 patient samples (Figure 4). Even with the small number of patient samples, taking average of the four samples, the overall...
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**Discussion**

Our study provided in vitro, in vivo, and ex vivo evidence of the role of TSY-1 in stimulating telomerase activity as well as telomere length in cultured CD34+ stem cells, CD4+ T cells, 3 of 4 normal T-cells from healthy individuals, and 3 of 4 bone marrow samples from patients with MDS experiencing refractory anemia. The effect of TSY-1 on telomerase activity or telomere length correlated well with its effect on increasing hematopoiesis observed in the immune-mediated AA animal model. Thus, it is possible that TSY-1 can help reduce the harmful effects of AA through regulating telomere length and/or telomerase activity.

There are numerous causes of bone marrow deficiency, which can be either congenital or acquired. The most severe form that is difficult to treat is AA. AA is a rare disease; almost half of the cases occur during the first three decades of life. The incidence is two cases per million per year in Western countries and about two to
three fold higher in Asia [20]. In its severe form, which is characterized by the destruction of hematopoietic stem cells causing pancytopenia and an empty bone marrow, AA is a life-threatening bone marrow failure disorder that if left untreated, results in very high mortality rate. Currently, AA treated with either immunosuppressive therapies or hematopoietic stem cell transplantations show only limited success [21].

The standard immunosuppressive therapy (IST) for AA is the combination of antithymocyte globulin (ATG) and cyclosporine (CsA) [22]. The benefits of this regimen as the initial therapy has been quantitated in systematic studies in the US, Japan and Europe: overall response is achieved in about two thirds of the patients; the cumulative incidence of relapse among responders is approximately 20-30% and clonal evolution occurs in about 10-15% of cases [23, 24]. Although the overall survival after IST for AA has improved significantly from the early 1970s to 2000, survival following IST has, however, failed to further improve during the past decade [25]. Subsequent efforts to improve beyond ATG/CsA have been mostly unsuccessfull [26]. Addition of G-CSF conferred no benefits in terms of survival, durable response, or reduction in infections [27].

An allogeneic bone marrow transplant (BMT) from an HLA-identical sibling remains the treatment of choice for patients with acquired SAA. The current 5-year survival for patients over 16 years of age receiving a BMT from an HLA-identical sibling is 74% [28]. Acute graft-versus-host-disease (GVHD) occurs in about 20-30% of patients and chronic GVHD in 30-40% [29]. Chronic GVHD has been a major cause of morbidity and mortality in patients who survived more than 2 years post-graft [29]. For patients without a matched sibling, unrelated bone marrow or peripheral blood stem cell transplantation from adult donors are usually performed. For the patients who cannot find a suitable unrelated bone marrow/peripheral blood donor in the world-wide network of registries, unrelated cord blood stem cell transplantation may offer an alternative option that have been explored; however, all stem cell transplantation remain high risk procedures with attendant morbidity and mortality.

It has only recently been recognized that abnormality of telomerase activity is a common pathway underlying bone marrow failure in constitutional and acquired aplastic anemia [16]. Short telomeres are seen in white blood cells in about a third of patients with AA [16]. Recently it has been shown that mutation in components of the telomerase complex is associated with low telomerase activity, progressive telomere erosion, and a deficient proliferative capacity of hematopoietic stem cells [10]. Patients with acquired aplastic anemia and short telomeres appear to have poorer responses to immunosuppression [8, 9]. Thus, modulation of telomerase activity may have a role in the treatment of telomere deficiency conditions such as telomerase-mutant acquired aplastic anemia.
TSY-1 has been used as a Chinese herb regime to treat patients with bone marrow deficiencies in China with some reported success [12, 13]. However, definitive clinical trial results are lacking, and like many Chinese herbs, the exact mechanisms of action are unknown. TSY-1 is produced by multiple herbs (the components are proprietary information of BTBTL) that are boiled in water. The product is an odorless watery fluid and it is characterized by low toxicity, as evidenced in our initial cytotoxicity study and pre-clinical animal study by others [12, 13].

While work is underway to isolate the biologically active components, as with all herbal medicine which may function through a combination of a number of components in therapeutically effective ratios, such an effort may be a long and tedious process. There may be multiple mechanisms that contribute to the effects that TSY-1 may exert on bone marrow deficiency. Our data demonstrate that, at least preliminary, TSY-1 may carry out its functions by modulating telomerase activity of hematopoietic cells. Further studies are warranted to explore the precise molecular mechanisms of how TSY-1 regulates telomerase activity and telomere length and also to test the TSY-1 in randomized control trials.

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