Thymosin α1 treatment reduces hepatic inflammation and inhibits hepatocyte apoptosis in rats with acute liver failure

XUELIANG YANG1, YUNRU CHEN1, JIAN ZHANG2, TIANTIAN TANG3, YING KONG1, FENG YE1, XI ZHANG1, XIAOJING LIU1 and SHUMEI LIN1

1Department of Infectious Diseases, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061; 2The Second Department of Gastroenterology, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068; 3Department of Infectious Diseases, Xi'an Children's Hospital, Xi'an, Shaanxi 710003, P.R. China

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Abstract. The present study aimed to evaluate whether thymosin α1 (Tα1) increases survival rates through the improvement of immunofunction and inhibition of hepatocyte apoptosis in rats with acute liver failure (ALF). A total of 25 rats were randomly divided into the control group (CG), the model group (MG) and the treatment group (TG). The CG received an intraperitoneal injection of saline (2 ml). The ALF rat model was established by the intraperitoneal injection of D-galactosamine (700 mg/kg) and lipopolysaccharide (10 µg/kg). The TG received an intraperitoneal injection of Tα1 (0.03 mg/kg) 1 h prior to and 30 min after modeling. An additional 63 rats were randomly divided into a CG (n=3), MG (n=30) and TG (n=30). Three rats were sacrificed at 3, 6, 9 and 12 h after establishment of the rat model to detect plasma alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), tumor necrosis factor (TNF)-α and interleukin-10 (IL-10). Liver samples were stained with hematoxylin and eosin and TUNEL, and reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to detect B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) in liver tissue. The results indicated that the survival rate of the TG was significantly higher compared with that of the MG at 24 h (P<0.05). Plasma ALT, AST and TBIL in the MG and TG increased over time (3-12 h), with ALT, AST and TBIL observed to be significantly lower in the TG compared with the MG at each time-point (P<0.05). Bax mRNA expression was significantly lower in the TG compared with the MG at each time-point (P<0.05), whereas Bcl-2 was significantly higher (P<0.05). In conclusion, Tα1 improved survival rates in an ALF rat model by downregulating TNF-α and upregulating IL-10, leading to the attenuation of hepatic inflammation and hepatocyte apoptosis.

Introduction

Acute liver failure (ALF) is a heterogeneous syndrome, which results in rapid deterioration of liver function with coagulopathy and encephalopathy, and induces systemic inflammation and multiple organ failure (1,2). The primary causes of ALF (including, hepatitis B virus infection, acute viral hepatitis, alcohol and hepatotoxic drugs) are variable and the mortality rate is high, ~60-80% of individuals suffer mortality after contracting ALF (3,4). Liver transplantation is the only effective therapy, but financial burden, graft shortage and possible life-threatening complications for donors present important limitations (5). Therefore, ALF continues to lack a widely available and effective therapy.

Immune system imbalance serves a critical function in the course of ALF (6,7). In particular, pro-inflammatory and anti-inflammatory cytokine imbalance in the liver and circulation triggers exaggerated immune response and induces adverse outcomes in ALF (3). During the progression of ALF, high levels of pro-inflammatory cytokines trigger the synthesis and release of anti-inflammatory cytokines. These anti-inflammatory cytokines inhibit pathogenic inflammation, but also suppress immune function, resulting in the development of a compensatory anti-inflammatory response (3). The
relationship between pro-inflammatory and anti-inflammatory cytokine responses is complex (8).

Immune deregulation is now recognized as being critical in ALF pathogenesis, and immune modulation has become a key aspect of ALF treatment (9,10). Thymosin α1 (Tα1), a 28-amino acid peptide, has multiple biological activities. Tα1 was originally considered to primarily increase T-lymphocyte function, and has been used in the treatment of chronic cancer, immune deficiencies and hepatitis B virus infection (11-13). However, Tα1 has also been identified to act as an endogenous regulator of innate and adaptive immune systems (14). Tα1 serves a unique function in balancing pro-inflammatory and anti-inflammatory cytokine production through the regulation of distinct Toll-like receptors (TLRs) on different dendritic cell subsets (15). Immune system deregulation serves a critical function in the course of sepsis. Tα1 has exhibited beneficial effects in late-stage clinical trials for the treatment of sepsis (16,17). However, to the best of our knowledge, whether Tα1 is able to regulate the immune system in ALF has not yet been investigated.

In the present study, a rat model of D-galactosamine hydrochloride (D-GalN)/lipopolysaccharide (LPS)-induced ALF was used in order to evaluate the efficacy of Tα1 and its mechanism of action. More precisely, the present study aimed to quantify the Tα1-induced pro-inflammatory and anti-inflammatory effects and the expression of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) associated with hepatocyte apoptosis and to evaluate the potential therapeutic interest of Tα1 for ALF.

Materials and methods

Animals and diets. A total of 88 specific-pathogen-free 6-week-old male Sprague-Dawley rats (weighing 180-220 g) were purchased from the Laboratory Animal Center of Xi’an Jiaotong University Health Science Center (Xi’an, China). Rats were housed at a temperature of 23-25˚C with a 12-h light/dark cycle and 60-70% humidity. Standard rodent food and water were supplied ad libitum and rats were housed for 3 days prior to the experiment to enable them to acclimatize to their environment.

Drugs and reagents. Tα1 was purchased from SciClone Pharmaceuticals, Inc. (Foster City, CA, USA). LPS and D-GalN were both purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), and dissolved in aseptic normal saline. Rabbit anti-mouse Bcl-2 (cat. no. ab59348), Bax (cat. no. ab53154) and GAPDH (cat. no. ab8245) were supplied by Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (cat. no. TA130015) was purchased from OriGene Technologies, Inc. (Beijing, China). ELISA kits for interleukin-10 (IL-10; cat. no. EA100170) and tumor necrosis factor-α (TNF-α; cat. no. EA101768) were purchased from OriGene Technologies, Inc. The In-Situ Apoptosis Detection kit was obtained from Promega Corporation (Madison, WI, USA). Total RNA Extraction kits were purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). The Sensiscript RT kit was purchased from Qiagen, Inc. (Valencia, CA, USA). SYBR Green Master mix was obtained from Applied Biosystems (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Animal grouping and drug administration. A total of 88 rats were randomly divided into two groups. One group of 25 rats was randomly subdivided into a control group (CG; saline, n=5), model group (MG; D-GalN/LPS, n=10) and treatment group (TG; Tα1/D-GalN/LPS, n=10). Survival rates were recorded for 24 h, then all surviving rats were sacrificed. The second group (n=63) was randomly subdivided into CG (n=3), MG (n=30) and TG (n=30). Three rats from the MG and TG groups were sacrificed at 3, 6, 9 and 12 h after D-GalN injection, and 3 rats from the CG were sacrificed at 3 h following saline injection. Rats in the CG group received an intraperitoneal injection of 2 ml saline. Rats in the MG group received intraperitoneal injection of D-GalN (700 mg/kg) and LPS (10 μg/kg) to induce ALF. Rats in the TG group received an intraperitoneal injection of Tα1 (0.03 mg/kg) 1 h before and 30 min after the establishment of the ALF model. All animal experiments were approved by the Ethics Committee of Xi’an Jiaotong University (Xi’an, China) in accordance with the guidelines of the China Laboratory Animal Management Committee.

Sample collection. Blood samples (~2 ml) were obtained by cardiac puncture in rats under 10% chloral hydrate anesthesia (300 mg/kg, intraperitoneally), and then centrifuged at 1,200 x g for 10 min at 25˚C. The supernatant was tested for the biochemical indices TNF-α and IL-10. The rats were sacrificed by cervical dislocation immediately after blood collection, and a portion of liver tissue was fixed in 6% paraformaldehyde at 4˚C for 24 h in PBS for pathological examinations. Another portion of liver tissue was frozen in liquid nitrogen for subsequent measurement of Bax and Bcl-2 expression.

Liver function tests. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) levels were determined using an LST008 Biochemistry Analyzer (Hitachi, Ltd., Tokyo, Japan).

Histopathology. Liver tissue, fixed in 6% paraformaldehyde in PBS, was dehydrated using ethyl alcohol. Ethyl alcohol in the tissues was then eliminated with xylene, and the liver sections embedded in paraffin for sectioning. The 5-μm-thick sections were stained with hematoxylin for 20 min and eosin for 3 min at 25˚C and a light microscope was used to examine the sections.

TUNEL assay. Fixed liver tissues were permeabilized with 1% Triton X-100 for 10 min. TUNEL staining was conducted with an In-Situ Apoptosis Detection kit, according to the manufacturer’s instructions and the results were examined under a light microscope. The percentage of TUNEL-positive liver cells was calculated manually following observation. Five higher magnification images were selected to obtain a quantitative analysis of the positive cells, and the apoptotic index (AI) was calculated as follows: AI (%) = apoptotic cell number/total cell number x 100.

ELISA assay. Plasma levels of TNF-α and IL-10 were measured with ELISA kits, according to the manufacturer’s instructions.
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR for the analysis of Bax and Bcl-2 was performed as previously described (18). Briefly, total RNA was extracted from liver tissue using RNA Extraction kits. mRNA was converted to cDNA using a Sensiscript RT kit according to the manufacturer's instructions and qPCR was performed using SYBR-Green Master mix, according to the manufacturer's instructions. The sequences of the primers used are presented in Table I. β-actin was used as an internal control and the 2−ΔΔCq method was used to quantify the results (19).

Western blot analysis. Liver tissue was homogenized in lysis buffer (cat. no. 78510) with protease inhibitor (cat. no. 78443) (both from Thermo Fisher Scientific, Inc.) by sonication. Protein concentration was determined using Pierce™ BCA assay (cat. no. 23225; Thermo Fisher Scientific, Inc.) Total lysate (50 µg/lane) was separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% milk for 1 h at 25°C and incubated with primary antibodies against Bcl-2 (dilution 1:500), Bax (dilution 1:500) and GAPDH (dilution 1:1,000), for 24 h at 4°C. After washing, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (dilution 1:5,000) at room temperature for 2 h. Protein bands obtained were visualized using an enhanced chemiluminescence system (cat. no. 35055; Thermo Fisher Scientific, Inc.). The protein bands were detected using the Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Survival was evaluated using life tables constructed from survival data with Kaplan-Meier plots. The comparisons with the model group were assessed using analysis of variance followed by post hoc multiple comparison tests (Tukey’s) at each time-point. P<0.05 was considered to indicate a statistically significant difference.

Results

Ta1 treatment improves survival in ALF. As shown in Fig. 1, all rats in the CG survived for 24 h, whereas the number of animals that died increased over time (from 3 to 24 h) in the MG and TG. The survival rate of the rats was significantly higher in the TG as compared with the MG (60 vs. 30%, P=0.0248).

Ta1 reduces liver injury in ALF. As shown in Tables II-IV, the plasma levels of ALT, AST and TBIL were significantly higher in the TG and MG compared with the CG at all time-points (P<0.05). Furthermore, the levels in the TG were significantly lower compared with the MG at all time-points (P<0.05), with the exception of TBIL at 3 h.

Typical hepatic histopathological features are presented in Fig. 2. Liver samples from the control rats exhibited an intact hepatic structure with normal hepatic lobule, and an absence of hepatocellular necrosis, hemorrhage or inflammatory cell infiltration (Fig. 2C). By contrast, notable damage was observed in the MG (Fig. 2A) and TG (Fig. 2B) samples. Evident destruction of architecture was observed, with a large number of apoptotic and necrotic cells in the two groups. In addition, infiltrating cells and congested red blood cells in the sinusoids were observed. Furthermore, the features of ALF were aggravated over time (3-12 h) in the MG and TG. Notably, in the Ta1-treated rats, the hepatocytes appeared healthier, with fewer apoptotic cells, as compared with those from the MG at the same time-points.

Ta1 inhibits pro-inflammatory TNF-α and promotes anti-inflammatory IL-10. Plasma TNF-α (Fig. 3A) and IL-10 (Fig. 3B) levels were significantly higher in the MG and TG compared with the CG at 3 h (P<0.05). Furthermore, the levels of TNF-α and IL-10 increased in the MG and TG at each time-point from 3 to 12 h. The level of TNF-α was significantly lower in the TG compared with the MG at each time-point (3 h, P<0.001; 6 h, P<0.001; 9 h, P=0.005; 12 h, P<0.001), whereas IL-10 was significantly higher in the TG compared with the MG (3 h, P=0.008; 6 h, P=0.003; 9 h, P=0.002; 12 h, P=0.009).

Ta1 inhibits hepatocyte apoptosis in ALF. As shown in Fig. 4, apoptotic liver cells were observed in the MG (Fig. 4A) and TG (Fig. 4B). The liver cell AI was significantly higher in the MG and TG compared with the CG at each time-point. The liver cell AI in the MG and TG increased at each time-point from 3 to 12 h (Fig. 4C). The AI in the TG was significantly lower

Table I. Primer sequences for polymerase chain reaction.

| Genes     | Primer                                       |
|-----------|----------------------------------------------|
| β-actin   | F: 5'-TCTGTGTGGATGTTGCTCTTA-3' R: 5'-CTGCTTGCTGATCACATCTG-3' |
| Bcl-2     | F: 5'-GGGATGCCTTTGGAACATATG-3' R: 5'-TGACCCGGCTTCAGAGACA-3' |
| Bax       | F: 5'-GACACCTGAGCTGACCTTGA-3' R: 5'-GACACTCGCTCAGCTTTTGT-3' |

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.
compared with that in the MG at 6, 9 and 12 h (6 h, P<0.001; 9 h, P=0.002; 12 h, P<0.001).

To confirm the apoptotic changes in the liver, Bax and Bcl-2 were investigated in ALF with or without Tα1 treatment, using RT-qPCR (Fig. 5A and B) and western blot analysis (Fig. 5C and D). Bax mRNA expression was significantly increased in the TG and MG compared with the CG at 3 h (Fig. 5A). The expression of Bax mRNA (Fig. 5A) and Bax protein (Fig. 5C) increased over time, from 3 to 12 h. The mRNA expression of Bax was significantly higher in the MG compared with the TG at each time-point (3 h, P<0.005; 6 h, P<0.001; 9 h, P=0.018; 12 h, P=0.01).

**Discussion**

The present study aimed to investigate the effects of Tα1 in a rat model of ALF, and to establish an experimental basis for the administration of Tα1 in the treatment of ALF. The results demonstrated that administration of Tα1, following the development of ALF, decreased plasma levels of ALT, AST, TBIL and TNF-α. Tα1 administration resulted in significantly higher plasma IL-10, together with reduced hepatic histological damage. Tα1 administration also improved the survival rates in this ALF rat model, suggesting that Tα1 exerted an overall beneficial effect by reducing the inflammatory reaction, decreasing cell damage and enhancing immune function in ALF rats.

### Table II. Measurements of ALT values at each time-point.

| Group | 3 h     | 6 h     | 9 h     | 12 h    |
|-------|---------|---------|---------|---------|
| CG    | 33.33±4.84 |         |         |         |
| MG    | 1,379.33±4.33<sup>a</sup> | 2,894.50±5.21<sup>a</sup> | 4,991.97±553.30<sup>a</sup> | 9,297.43±81.54<sup>a</sup> |
| TG    | 985.50±3.80<sup>b</sup> | 1,863.34±36.89<sup>b</sup> | 2,583.35±174.73<sup>b</sup> | 6,187.67±182.89<sup>b</sup> |

Data are presented as the mean ± standard deviation. *P<0.05 vs. CG; †P<0.05 vs. MG. ALT, alanine aminotransferase; CG, control group; MG, model group; TG, treatment group.

### Table III. Measurements of AST values at each time-point.

| Group | 3 h     | 6 h     | 9 h     | 12 h    |
|-------|---------|---------|---------|---------|
| CG    | 34.67±1.53 |         |         |         |
| MG    | 847.40±43.34<sup>a</sup> | 1,321.36±29.26<sup>a</sup> | 3,442.00±409.67<sup>a</sup> | 6,778.00±91.60<sup>a</sup> |
| TG    | 622.33±58.77<sup>b</sup> | 999.71±99.03<sup>b</sup> | 2,159.00±179.41<sup>b</sup> | 4,415.00±171.56<sup>b</sup> |

Data are presented as the mean ± standard deviation. *P<0.05 vs. CG; †P<0.05 vs. MG. AST, aspartate aminotransferase; CG, control group; MG, model group; TG, treatment group.

### Table IV. Measurements of TBIL values at each time-point.

| Group | 3 h     | 6 h     | 9 h     | 12 h    |
|-------|---------|---------|---------|---------|
| CG    | 3.76±0.45 |         |         |         |
| MG    | 7.07±0.61<sup>a</sup> | 11.60±1.08<sup>a</sup> | 16.30±0.87<sup>a</sup> | 22.50±2.11<sup>a</sup> |
| TG    | 6.97±0.60<sup>b</sup> | 9.23±0.49<sup>b</sup> | 11.37±0.50<sup>b</sup> | 14.67±0.45<sup>b</sup> |

Data are presented as the mean ± standard deviation. *P<0.05 vs. CG; †P<0.05 vs. MG. TBIL, total bilirubin; CG, control group; MG, model group; TG, treatment group.
Thymosin α1 has been clinically demonstrated to exert an immune modulatory activity by promoting the maturation of T cells and natural killer cells, activating dendritic cells (20-22), increasing cytokine production, and modulating major histocompatibility complex class I surface molecules and tumor antigens (23,24). Therefore, Thymosin α1 has been used to treat hepatitis B, resistance to infection and breast cancer (13,25,26). Thymosin α1 is a peptide that affects multiple immune subsets associated with immune suppression (27). Furthermore, previous studies have reported that Thymosin α1 serves a critical function in balancing pro- and anti-inflammatory cytokine production through the involvement of distinct TLRs acting on different dendritic cell subsets and the MyD88-dependent signaling pathway (28). Thymosin α1 may attenuate tissue injury and reduce the mortality rate through reducing the release of inflammatory factors and cytokines, and increasing IL-10 to control inflammation (21,29,30).

Increased necrosis and apoptosis of hepatocytes is believed to release various inflammatory cytokines in ALF (31).
Figure 3. Thymosin α1 treatment inhibits pro-inflammatory TNF-α and promotes anti-inflammatory IL-10. Plasma (A) TNF-α and (B) IL-6 levels. Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 vs. the CG group. TNF-α, tumor necrosis factor-α; IL-10, interleukin-10; CG, control group; MG, model group; TG, treatment group.

Figure 4. Thymosin α1 treatment inhibits hepatocyte apoptosis in D-galactosamine hydrochloride/lipopolysaccharide-induced ALF. Liver tissues were harvested at 3, 6, 9 and 12 h after the establishment of a rat model of ALF and a TUNEL assay was performed (magnification, x400). Representative images from 3 rats/group were selected. (A) MG at a-1, 3 h; a-2, 6 h; a-3, 9 h; and a-4, 12 h. (B) TG at b-1, 3 h; b-2, 6 h; b-3, 9 h; and b-4, 12 h. (C) CG 3 h. (D) Quantification of liver cell apoptosis. Data are expressed as the mean ± standard error of the mean (n=3). *P<0.05 vs. the CG group. ALF, acute liver failure; CG, control group; MG, model group; TG, treatment group; AI, apoptotic index.
TNF-α serves a key function in liver injury and hepatic apoptosis (32,33). Pro-inflammatory TNF-α expression is markedly upregulated in ALF, which is critical to reducing the survival rate of mouse models of ALF induced by D-GalN/LPS (34). The present study identified that TNF-α and AI in the TG were lower compared with those in the MG following Tα1 treatment, suggesting that Tα1 reduced TNF-α levels through the inhibition of apoptosis to counteract liver failure. Furthermore, the rat mortality rate decreased with Tα1 treatment, in line with the changes in TNF-α.

IL-10 is a key cytokine derived from T cells, macrophages and monocytes, which is able to inhibit the production of multiple immune active cytokines and to alleviate tissue damage (35,36). A shift to IL-10 production occurs following the pro-inflammatory phase and this shift is initiated by high TNF-α levels (37). In a previous study, although serum concentrations of TNF-α and IL-10 were increased in ALF, treatment with IL-10 resulted in normalization of aminotransferase levels, improved liver histology and reduced fibrosis (38). In addition, administration of recombinant IL-10 prior to the establishment of ALF has been demonstrated to reduce the production of these pro-inflammatory cytokines and improve liver injury (39). The present study identified that the plasma levels of IL-10 were higher in the TG compared with the MG after Tα1 treatment, indicating that Tα1 promoted the anti-inflammatory cytokine IL-10 to suppress excessive immune response (particularly inflammatory TNF-α) and prevent further liver cell apoptosis. Tα1 can also affect the relative ability of dendritic cells to balance T helper cells and regulatory T cells to stimulate IL-10 in vitro and in vivo (28). IL-10 in turn negatively regulates the immune response and prevents a large excess of cytokines, known as a ‘cytokine storm’ (40).

However, the present study should be regarded as a pilot study, as it only investigated plasma TNF-α and IL-10, and the results of immunomodulation in animal models do not directly translate to clinical settings. Further investigations are required at the biochemical level (including additional liver function and cytokine testing) and molecular level (such as gene expression studies) in order to fully understand the immunological mechanisms underlying ALF and the effects of Tα1.

In conclusion, the use of immunomodulatory agent Tα1 may have beneficial effects in ALF by alleviating inflammatory responses and reducing cell, tissue and organ damage. Therefore, the clinical application of immunomodulation therapy in ALF treatment deserves further investigation.

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