Original Article
Effects of combined thymosin and hydrocortisone on immune response in septic mice

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Received May 13, 2015; Accepted July 3, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: This study is to investigate the effects of the thymosin α1 (Tα1) and hydrocortisone (HC) combination treatment on the immune responses in septic mice. According to different treatments, mice were divided into the control group (n = 18), the sepsis model group (n = 18), the Tα1 group (n = 18), the HC group (n = 18), and the Tα1+HC group (n = 18). Septic mouse model was established by the intraperitoneal injection of lipopolysaccharide (LPS). At 72 h after modeling, flow cytometry was used to analyze the dendritic cell (DC) numbers in peripheral blood and the expressions of MHC II and CD86. Tumor necrosis factor-α (TNF-α) level was measured by ELISA. Treatments of Tα1 and/or HC dramatically increased the survival rates of LPS-induced septic mice. Flow cytometry showed that, the DC numbers in peripheral blood were significantly decreased in the sepsis model group, which could be dramatically elevated by Tα1 treatment alone and in combination with HC (Tα1+HC). However, the DCs were undetectable in the HC group. In addition, the MHC II expression level was decreased in the sepsis model group, which was further declined in the Tα1 and Tα1+HC groups. The expression level of CD86 was elevated in the model group, which could be significantly down-regulated by the treatments of Tα1 and Tα1+HC. ELISA showed that, the peripheral blood TNF-α level in the HC group was lower than in the sepsis model group. Compared with the sepsis model group, the TNF-α levels were significantly elevated in the Tα1 and Tα1+HC groups. Tα1 and HC combination treatment could improve the immune function and regulate the inflammatory response to increase the survival rates of LPS-induced septic mice.

Keywords: Sepsis, thymosin, hydrocortisone, immune response, dendritic cells

Introduction

Sepsis is one of the primary causes of death in critically ill patients in the intensive care unit (ICU), which is the result of excessive host inflammatory responses caused by infection [1]. In recent years, the sepsis-induced multiple organ dysfunction syndrome (MODS) has attracted increasing attention [2], and the disease pathogenesis and especially the treatments of the disease have been intensively studied.

Thymosin α1 (Tα1) is a potent immune modulator released by the thymus gland, which has effects on immunodeficiency, cancer, and viral infectious diseases [3]. Tα1 could be used as an immune response regulator in the treatment of critically ill patients [4]. On the other hand, glucocorticoids have been widely used to treat septic patients as an adjunctive therapy. However, the survival rate cannot be improved by the hormone treatment [5]. Besides, the application of glucocorticoids in septic patients may even lead to a secondary infection and increase the mortality [6, 7].

Recent studies indicate that the body’s immunity can be influenced via the regulation of dendritic cells (DCs) [8, 9]. DCs are important players in the specific and non-specific immune system [10, 11]. Because of the high expression of MHC II on the cell surface and the ability to activate naive T cells, DCs represent professional antigen-presenting cells [11]. It has been shown that the modulation of in vivo DCs can affect the immune system in mice [12-
Moreover, clinical studies have found that patients receiving effective treatments have obviously higher levels of DCs, and the survival rate might be associated with the status of DCs [16, 17]. Profound DC depletion could be observed in septic patients, as well as animal models [12-15]. In this study, septic mouse model was established, and treated with Tα1 and/or hydrocortisone (HC). The effects of the treatments on DCs and the immune response in these mice and the related mechanisms were investigated.

Materials and methods

Animal modeling and grouping

A total of 90 male C57BL/6J mice, weighing 20-25 g, were provided by the Experimental Animal Center of the First Teaching Hospital of Xinjiang Medical University. Septic mouse model was established by the intraperitoneal injection of 1 mL lipopolysaccharide (LPS) into mice, as previous described [18].

The mice were randomly divided into the following groups: (1) the control group (n = 18), in which mice were injected with 1 mL saline instead of LPS; (2) the sepsis model group (n = 18), in which mice were injected with LPS to induce sepsis (10 mg/kg body weight); (3) the Tα1 group (n = 18), in which LPS-induced septic mice were treated with 1 mL Tα1 (1 mg/kg body weight); (4) the HC group (n = 18), in which LPS-induced septic mice were treated with 1 mL HC (5 mg/kg body weight); and (5) the Tα1+HC group (n = 18), in which LPS-induced septic mice were treated with 1 mg/kg Tα1 and 5 mg/kg HC. At 72 h after drug administration, the mice were sacrificed by cervical dislocation, and the blood samples were collected into tubes containing sodium citrate and heparin.

Flow cytometry

Totally 50 μL blood sample from each group were divided into four groups for antibody tests. In Group A, APC-labeled anti-IgG and PE-labeled anti-IgG2b isotype control antibodies were sequentially added, which was used as the control group for flow cytometry. In Group B, APC-labeled anti-CD11c and PE-labeled anti-CD86 monoclonal antibodies were sequentially added. In Group C, APC-labeled anti-CD11c and PE-labeled anti-MHC II monoclonal antibodies were sequentially added. In Group D, FITC-labeled anti-CD8a, PE-labeled anti-CD4, and APC-labeled anti-CD11c antibodies were added. All the samples were thoroughly mixed, and then incubated in dark at room temperature for 15 min. After adding hemolytic agent, the samples were incubated in dark for another 10 min, and washed twice with PBS. The samples were detected by a flow cytometer (FACSCalibur™; BD Biosciences, San Jose, CA, USA), and the data were analyzed with the Cell Quest software (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected at 72 h by centrifugation. The TNF-α levels in the serum samples were measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using the SPSS 13.0 software. ANOVA was used for the analysis of the pair-wise comparison. P < 0.05 was considered statistically significant.

Results

Tα1 and/or HC treatments increase survival rates of septic mice

To investigate the effects of Tα1 and/or HC treatments on septic mice, the survival rates were obtained in these mice during 72 h after modeling (LPS injection). As shown in Figure 1, our results indicated that, the survival rate in the model group started to decline at only 3 h after LPS injection, and all the mice were dead at 24 h after injection. However, the treatments of Tα1 and/or HC dramatically increased the survival rates in these model mice, and the most obvious effect was observed in the combination treatment group. At 72 h after LPS injection, the survival rates in the Tα1 group and the HC group were 55.6% and 27.8%, respectively, which were significantly lower than the survival rate of 83.3% in the Tα1+HC group (P < 0.05) (Figure 1). These results suggest that the treatments of Tα1 and/or HC could significantly increase the survival rates of these LPS-induced septic mice.
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![Graph showing survival rates over time]

**Figure 1.** Tα1 treatment alone and Tα1+HC combination increased the survival rates of septic mice. During 72 h after modeling (LPS injection), the survival rates were calculated for the control, LPS-induced sepsis model, Tα1-treated, HC-treated, and combination-treated groups.

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**To investigate the effects of Tα1 and/or HC treatments on the immune response in septic mice, the numbers of DCs in peripheral blood were counted with flow cytometry. Our results showed that, there was no significant difference in the number of DCs between the control group and the LPS-induced sepsis model mice (P > 0.05) (**Figure 2**). Moreover, the DC numbers in these sepsis model mice were dramatically elevated by the treatments of Tα1 alone and Tα1+HC combination (both P < 0.05). However, the DCs were undetectable in the HC group (**Figure 2**). These results suggest that Tα1 treatment alone and the Tα1+HC combination treatment could significantly elevate the numbers of DCs in peripheral blood, i.e., enhance the immune response, in the LPS-induced septic mice.

**Tα1 treatment alone and Tα1+HC combination treatment could significantly elevate the numbers of DCs in peripheral blood, i.e., enhance the immune response, in the LPS-induced septic mice.**

![Bar chart showing DC numbers]

**Figure 2.** Tα1 treatment alone and Tα1+HC combination increased the DC numbers in peripheral blood in septic mice. The numbers of DCs in peripheral blood were counted with flow cytometry at 72 h after modeling. Compared with the control group, *P < 0.05; compared with the LPS model group, #P < 0.05.

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**Ta1 treatment alone and Ta1+HC combination increase DC numbers in peripheral blood in septic mice**

To further investigate the effects of Tα1 and/or HC treatments on the immune response, the MHC II and CD86 cell surface antigen expression on the DCs was detected by flow cytometry. In consistence with the DC counting in peripheral blood, the expression levels of MHC II and CD86 in the HC group could not be accurately detected. Therefore, only the results for the control, model, Tα1, and Tα1+HC groups were presented. Our results showed that, compared with the control group, the MHC II expression level was decreased in the LPS-induced sepsis model group (P < 0.05), which was further declined in the Tα1 and Tα1+HC groups (compared with the control group, P < 0.05) (**Figure 3A**). On the other hand, compared with the control group, the expression level of CD86 was significantly elevated in the model group (P < 0.05). However, the treatments of Tα1 and Tα1+HC significantly down-regulated the expression levels of CD86 in these mice (P < 0.05) (**Figure 3B**). These results suggest that Tα1 treatment alone and Tα1+HC combination treatment could alter the expression levels of immunoregulatory proteins in the LPS-induced septic mice.

**Tα1 treatment alone and Tα1+HC combination alter immunoregulatory protein expression levels in septic mice**

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**Tα1 treatment alone and Tα1+HC combination alter immunoregulatory protein expression levels in septic mice**

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To confirm the effects of Tα1 and/or HC treatments on the immune response, the serum TNF-α level was detected by ELISA. Our results...
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Figure 3. Tα1 treatment alone and Tα1+HC combination altered the expression levels of immunoregulatory proteins. The expression levels of immunoregulatory proteins, MHC II (A) and CD86 (B), on DC surface were detected by flow cytometry at 72 h after modeling. Compared with the control group, *P < 0.05; compared with the LPS model group, †P < 0.05.

Figure 4. Tα1 treatment alone and Tα1+HC combination increased serum TNF-α level in septic mice. Serum TNF-α level at 72 h after modeling was detected by ELISA. Compared with the control group, *P < 0.05; compared with the LPS model group, †P < 0.05.

showed that, the serum TNF-α level in the HC group was lower than that in the model group. On the other hand, compared the LPS-induced sepsis model group, the serum TNF-α levels were significantly elevated in the Tα1 and Tα1+HC groups (both P < 0.05) (Figure 4). These results suggest that Tα1 treatment alone and Tα1+HC combination treatment could significantly increase the serum TNF-α level in the LPS-treated septic mice.

Discussion

It has been first reported by Tisley et al. [13] that the spleen DCs were declined, especially the follicular DCs, in septic mice established by the cecal ligation and puncture (CLP) method. Moreover, Hotchkiss et al. [14] have also reported the spleen DC loss through autopsy in septic patients. Furthermore, Guisset et al. [15] have shown that the DC loss in peripheral blood of septic patients was closely related to the mortality rate, which was mainly caused by cellular apoptosis. In addition, numerous experimental and clinical studies have confirmed the association of the alteration in DCs with the prognosis of the septic patients [12-15]. Clinical data have shown that the DC numbers in peripheral blood of septic patients ending up with death were obviously lower than that of the surviving patients, which is associated with the SAPSII scores of the patients [14]. In this study, our results showed that, at 72 h after modeling, the survival rates in the Tα1 and HC groups were 55.6% and 27.8%, respectively. Moreover, the Tα1+HC combination treatment group showed the highest survival rate (83.3%) in all these groups. In addition, compared with the control group, the DC numbers in peripheral blood in the Tα1 and Tα1+HC groups were significantly increased, while the DCs were almost undetectable in the HC group at 72 h after modeling. Taken together, these results suggest that the survival rate of septic mice is increased along with the increasing DC numbers, and DCs might be important regulators of the immune system in sepsis. Glucocorticoids are actively involved
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in the immune activity [19, 20]. Our results showed that the DC numbers in peripheral blood in the HC group were obviously lower than in the other groups, implying that dexamethasone-caused DC loss might be an important mechanism through which the immune response was affected.

Thymosin has been widely used for the treatment of sepsis [21, 22]. It has been reported that DC is an important target of thymosin, and the proliferation of DCs can be stimulated by thymosin, through the TLR signaling pathway, activating the immune response [23]. Our results have shown that, after treated with Tα1 or Tα1+HC, the DC numbers in peripheral blood in septic mice were significantly increased, which suggested that thymosin obviously enhanced the immune response in these mice. On the other hand, we found that the expression levels of CD86 on the DCs at 72 h after modeling were slightly decreased in the Tα1 and Tα1+HC groups. Furthermore, the MHC II expression on the blood DCs was also significantly down-regulated in both these groups, suggesting that Tα1 may increase the replenishment of the DC pool. Since the DCs were almost undetectable in peripheral blood in the HC group, the expression levels of cell surface molecules MHC II and CD86 were also unable to be accurately detected.

TNF-α is one of the most important inflammatory mediators in stress response, which is always first synthesized and released [24]. Our results found that the TNF-α content showed increasing trends in the Tα1 and Tα1+HC groups, suggesting that the treatments of Tα1 and Tα1+HC could inhibit the release of TNF-α in septic mice, reduce inflammatory injuries, and protect organ functions. The increased DC numbers indicated that thymosin might induce DC pool repairing, and thus change the immune status of the body, reversing the immunosuppression and increasing the immunity.

In summary, our results showed that the combination of Tα1 and HC could improve the immune function and regulate the inflammatory response to increase the survival rates of LPS-induced septic mice. In addition to the traditional active anti-infection and correct shock treatment, combination of Tα1 and HC could be considered for the treatment of septic patients, which may help to regulate the immune status in these patients and improve the prognosis.

Acknowledgements

This work was supported by the School of Medicine of the First Affiliated Hospital of Shihezi University (No. YL2014-S012).

Disclosure of conflict of interest

None.

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