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Fas/FasL in the immune pathogenesis of severe aplastic anemia

C.Y. Liu, R. Fu, H.Q. Wang, L.J. Li, H. Liu, J. Guan, T. Wang, W.W. Qi, E.B. Ruan, W. Qu, G.J. Wang, H. Liu, Y.H. Wu, J. Song, L.M. Xing and Z.H. Shao

Department of Hematology, General Hospital, Tianjin Medical University, Tianjin, China

Corresponding author: Z.H. Shao
E-mail: shaozonghong_l@yeah.net

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ABSTRACT. Fas/FasL protein expression of bone marrow hematopoietic cells was investigated in severe aplastic anemia (SAA) patients. Fas expression was evaluated in $CD34^+$, $GlycoA^+$, $CD33^+$, and $CD14^+$ cells labeled with monoclonal antibodies in newly diagnosed and remission SAA patients along with normal controls. FasL expression was evaluated in $CD8^+$ cells in the same manner. In $CD34^+$ cells, Fas expression was significantly higher in the newly diagnosed SAA group (46.59 ± 27.60%) than the remission (6.12 ± 3.35%; $P < 0.01$) and control (8.89 ± 7.28%; $P < 0.01$) groups. In $CD14^+$, $CD33^+$, and $GlycoA^+$ cells, Fas levels were significantly lower in the newly diagnosed SAA group (29.29 ± 9.23, 46.88 ± 14.30, and 15.15 ± 9.26%, respectively) than in the remission (47.23 ± 31.56, 67.22 ± 34.68, and 43.56 ± 26.85%, respectively; $P < 0.05$) and normal control (51.25 ± 38.36, 72.06 ± 39.88, 50.38 ± 39.88%, respectively; $P < 0.05$) groups. FasL expression of $CD8^+$ cells was significantly higher in the newly diagnosed SAA group (89.53 ± 45.68%) than the remission (56.39 ± 27.94%; $P < 0.01$) and control (48.63 ± 27.38%; $P <0.01$) groups. No significant differences were observed between the remission and control

groups. FasL expression in $CD8^+$ T cells was significantly higher in newly diagnosed patients, and $CD34^+$, $CD33^+$, $CD14^+$, and $GlycoA^+$ cells all showed Fas antigen expression. The Fas/FasL pathway might play an important role in excessive hematopoietic cell apoptosis in SAA bone marrow. Furthermore, $CD34^+$ cells are likely the main targets of SAA immune injury.

Key words: Severe aplastic anemia; Fas/FasL; Apoptosis

INTRODUCTION

Severe aplastic anemia (SAA) immunopathogenesis is closely associated with the hyperactivity of T cell function, which damages bone marrow hematopoiesis. Its clinical manifestations include serious infections, anemia, and bleeding, and patients have a high risk of death. Currently, the immunosuppressive therapy of antilymphocyte globulin combined with cyclosporine A is generally adopted in clinical settings; however, 30% of patients do not receive effective treatment (Locasciulli et al., 2007). After treatment, alleviated patients have different reactions to immunosuppressive therapy with respect to extent and speed in the medication process. Therefore, the effector T cells that damage bone marrow hematopoiesis in SAA patients show heterogeneity. The reasons for this heterogeneity may be related with the apoptosis pathway of the damaged target cells. Understanding of the Fas system has contributed great progress to human programmed cell death and apoptosis research in recent years. The Fas system plays an important role in the immune system disease. This study aimed to investigate the Fas/FasL system of patients with SAA to further clarify SAA apoptosis of bone marrow hematopoietic cells.

MATERIAL AND METHODS

Subjects

Fifteen cases of newly diagnosed SAA patients were selected from March 2010 to March 2011 in our department, and these patients did not receive any treatment. This group included 9 males and 6 females with a median age of 27 (range = 16-42) years. All patients underwent T cell subset detection, T cell activation state detection, CD55-CD59 cell detection, morphological and histochemical analysis of bone marrow cells, bone marrow biopsy, bone marrow hematopoietic stem/progenitor cell culture, bone marrow mononuclear cells, Coombs test, and chromosome karyotype analysis. The diagnostic criteria were consistent with the standard SAA diagnostic criteria in blood disease diagnosis and efficacy (Zhang and Shen, 2007). Fifteen control subjects were selected from normal, healthy individuals, including 7 males and 8 females with a median age of 31 (range = 26-40) years. The research program was approved by the Ethics Committee of Tianjin Medical University, and all subjects provided informed consent.

Main reagents and instruments

PerCP-labeled mouse anti-human CD34, CD45, and CD8 monoclonal antibody, PE-

labeled mouse anti-human CD95 monoclonal antibody, FITC-labeled mouse anti-human CD34 monoclonal antibody, APC-labeled mouse anti-human CD33, CD14, GlycoA monoclonal antibody, and their respective isotype controls were purchased from the Becton Dickinson company (USA). The FACSCalibur flow cytometer was used (Becton Dickinson).

Research methods

Two milliliters of fresh heparin bone marrow fluid was collected, and 100 μL bone marrow fluid was added into the detector tube. CD45/CD14/CD33/Fas, CD45/GlycoA/Fas, CD34/Fas, CD8/FasL, and their 20 μL isotype control monoclonal antibodies were added to the test tube. Samples were incubated at 4°C for 30 min, and then 2 mL erythrocyte lysis buffer was added and mixed in the dark at room temperature for 10 min. After centrifugation at 1500 rpm for 5 min, the supernatant was removed, and 2 mL phosphate-buffered saline (PBS) solution was added. After another 1500 rpm centrifugation for 5 min, the supernatant was removed, washed two times with PBS, and filtered for the machine testing; 500,000 cells were collected per tube. The Diva analysis software was used, side scatter/forward scatter (SSC/FSC) were selected first for gating, the debris and dead cells were removed, and nucleated cells inside the door were selected for protein detection. The cells with low SSC and FSC were selected in the second gating, and the SSC/CD34 was used for the third gating. The $CD34^+$ cell colonies were selected (P3), and the Fas expression level of $CD34^+$ cell colonies was analyzed. The SSC/CD45 was used for the third gating, the $CD14^+$, $CD33^+$, $GlycoA^+$ colonies were selected (P3, P4, P5), and the Fas expression levels of $CD14^+$, $CD33^+$, and $GlycoA^+$ colonies were analyzed. The lymphocyte gate was set in the locating plot of FSC and SSC, and the P2 gate was set by $CD8^+$ cells in the locating plot of SSC, PerCP. The cells inside the P2 gate were set as the negative cross gate, and cell membrane FasL expression levels were detected.

Statistical analysis

The statistical analysis software SPSS 11.5 for Windows was used. Normally distributed numerical variables are reported as means ± standard deviation (SD). The difference between two groups was compared using the Student *t*-test. The skewed distributed data are reported with the median and was analyzed with the Wilcoxon test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Hematopoietic cell expression of Fas across various stages in bone marrow of SAA patients

The Fas protein expression level of the bone marrow $CD34^+$ cells in the newly diagnosed SAA group was 46.59 ± 27.60%, which was significantly higher than that of the remission group (6.12 ± 3.35%, $P < 0.01$) and the control group (8.89 ± 7.28%, $P < 0.01$). The difference was not statistically significant between the remission group and the normal control group ($P > 0.05$). The Fas protein expression levels of $CD14^+$, $CD33^+$, and $GlycoA^+$ in the newly diagnosed SAA group were 29.29 ± 9.23, 46.88 ± 14.30, and 15.15 ± 9.26%, respec-

tively, which were significantly lower than those of the remission group (47.23 ± 31.56, 67.22 ± 34.68, and 43.56 ± 26.85%, respectively, $P < 0.05$) and the normal control group (51.25 ± 38.36, 72.06 ± 39.88, and 50.38 ± 39.88%, respectively, $P < 0.05$) (Table 1). The difference was not statistically significant between the remission group and the normal control group ($P > 0.05$).

Table 1. Fas expression of the hematopoietic cells at various stages in bone marrow.

Group	N	$CD34^+$ cells	$CD14^+$ cells	$CD33^+$ cells	$GlycoA^+$ cells
Newly diagnosed group	15	46.59 ± 27.60[b]	29.29 ± 9.23[a]	46.88 ± 14.30[a]	15.15 ± 9.26[a]
Remission group	15	6.12 ± 3.35	47.23 ± 31.56	67.22 ± 34.68[a]	43.56 ± 26.85
Control group	15	8.89 ± 7.28	51.25 ± 38.36	72.06 ± 39.88	50.38 ± 39.88

Data are reported as means ± SD in percentage. The SAA group was compared with the control group. [a]$P < 0.05$. [b]$P < 0.01$.

FasL expression of bone marrow $CD8^+$ cells in SAA patients

FasL expression of the bone marrow $CD8^+$ cells in the newly diagnosed SAA group was 89.53 ± 45.68%, which was significantly higher than that in the remission group (56.39 ± 27.94%, $P < 0.01$) and the control group (48.63 ± 27.38%, $P < 0.01$).

DISCUSSION

Hematological and immunological studies of the pathogenesis of SAA have revealed that SAA is an effector T cell function hyperactivity-mediated and bone marrow-targeted autoimmune disease. SAA patients show severe infection, anemia, and bleeding as clinical manifestations. The peripheral blood cells are greatly reduced and the bone marrow hematopoietic function is nearly completely inhibited, resulting in a high risk of death. Antilymphocyte globulin/antithymocyte globulin-based intensive immunosuppressive treatment has achieved increased clinical efficacy in recent years (Shao, 2010; HBCMA, 2010). We also found positive effects with respect to reaction time and regularity in patients who underwent immunosuppressive therapy in the clinical course of treatment. Blood levels took 3 to 6 months to return to normal in patients with fast responses, whereas the process could take one or several years in patients with slow responses. The variation in hematopoietic recovery time and speed among patients suggests that the incidence of SAA shows a high degree of heterogeneity. The reasons for this heterogeneity may be related to the different mechanisms of cytotoxic T lymphocyte (CTL) immune damage to the specifically targeted bone marrow hematopoietic cells. Therefore, we studied the damage pathways of SAA bone marrow hematopoietic-targeted cells in order to further clarify the specific mechanisms of SAA immune injury in bone marrow hematopoietic cells.

The Fas/FasL system is one of the most important aspects of human apoptosis system regulation, and is closely related to the development of many diseases. The normal state of the Fas system maintains equilibrium of the body's metabolism and induces aging and abnormal apoptosis. By contrast, in the abnormal state of the Fas system, cells can escape normal immune surveillance and proliferate excessively, or apoptosis might increase due to excessive immune attacks (Randhawa et al., 2010). Several studies have found that Fas/FasL expression was abnormal in many apoptosis-related diseases, including autoimmune diseases, neoplastic diseases, inflammations, and infections (Randhawa et al., 2010). Fas was first identified in

mice by Japanese researchers. It is a protein component that can dissolve human cells and combine with FasL. It is also a member of the tumor necrosis factor receptor/nerve growth factor receptor superfamily, which belongs to the type I group of transmembrane receptor proteins consisting of three parts: the extracellular N-terminal, the transmembrane region, and the intracellular C-terminal. The amino acid sequence of the outer membrane is relatively conservative and plays a role in signal transduction, which is also known as the death domain as it can initiate an apoptosis signal after binding with FasL. Fas trimers form and combine with the Fas-associated protein along with the caspase-8 precursor through the death decided clusters to form a death-inducing signal transduction complex, which ultimately affects cell structure proteins, promotes cell degradation, and leads to apoptosis. Fas is expressed in a variety of cell surfaces under normal circumstances, including activated T cells, B cells, monocytes, and granulocytes, to regulate the proliferation and clearance of T and B cells. It is also one important way in which the CTL exerts its cytotoxic effects. CTL is activated and it expresses FasL after being stimulated by a specific antigen, and then combines with Fas on the target cell surface to initiate the death program and apoptosis of target cells (Bohana-Kashtan and Civin, 2004). In malignant tumor diseases, the expression of Fas might be reduced on the surface of tumor cells in order to escape the removal functions of immune cells, enabling tumor cells to seize proliferation opportunities resulting in disease. Fas expression increases in autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, and Grave's disease, which causes immune cells to identify and kill their own organizations (Ehrenschwender and Wajant, 2009; Pryczynicz et al., 2010; Otsuki et al., 2011). The results of the present study showed that FasL expression on the $CD8^+$ T cell surface was significantly higher in SAA patients compared to the normal population. FasL expression levels returned to normal after immunosuppressive treatment. This suggested that the CTL might increase FasL expression to induce bone marrow hematopoietic apoptosis, which eventually leads to hematopoietic failure.

Furthermore, we analyzed the expression of the Fas antigen on various hematopoietic faculties and stages of blood cells in the bone marrow. We found Fas antigen expression in the $CD34^+$, $CD33^+$, $CD14^+$, and $GlycoA^+$ cells in bone marrow of SAA patients. In addition, Fas expression of $CD34^+$ cells in SAA patients was significantly higher than that in the normal population. Fas antigen expressions in erythroid, granulocyte, and monocyte cells were all significantly lower than those in normal controls, suggesting that compared with other kinds and stages of bone marrow hematopoietic cells, $CD34^+$ cells are relatively more susceptible to CTL immune attack. Due to the compensatory effects, Fas expression in other late-stage cells decreased to levels lower than the normal state. These effects caused delayed apoptosis and extended the life cycle to compensate for the immune damage, which was expressed as insufficient hematopoietic cells in various kinds and stages. In addition, Fas antigen expression returned to normal levels after immunosuppressive therapy in SAA patients with remission, which further suggested that Fas/FasL plays an important role in bone marrow hematopoietic cells of SAA immune injury.

In summary, this study found that the excessive apoptosis of bone marrow hematopoietic cells in patients with SAA might be due to recognition of the Fas expression antigen by the FasL expression CTL. Fas expression in $CD34^+$ cells significantly increased, indicating that $CD34^+$ cells may be the main target cells of SAA immune injury. More in-depth investigations of Fas/FasL and its mediated apoptosis signal should help to clarify the specific mechanisms of bone marrow hematopoietic cell injury under SAA, and reveal specific SAA damage categories and characteristics of the target cells. The present study also provided a new direction and

framework for the clinical treatment of SAA. Nevertheless, the biological characteristics of $CD34^+$ cells need further study to explore the real target of SAA immune attack and to improve SAA immune pathogenesis.

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