Journal of Huazhong University of Science and Technology [Med Sci]
DOI10.1007/s11596-007-0512-4
27 (5): 524-527, 2007

Changes of $CD4^+CD25^+$ Regulatory T Cells in Patients with Acute Coronary Syndrome and the Effects of Atorvastatin

HU Zhenping (胡珍娉)[1], LI Dazhu (李大主)[2#], HU Yingfeng (胡英锋)[2], YANG Keping (杨克平)[2]
[1]*Department of Geriatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Cardiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

Summary: The function of $CD4^+CD25^+$ regulatory T lymphocytes (Treg) in patients with acute coronary syndrome (ACS) and the effects of atorvastatin were investigated. Forty-eight patients with ACS were randomly divided into two groups: group C receiving conventional therapy (n=24), and group C+A receiving conventional therapy+atorvastatin (10 mg/day, n=24). T lymphocytes from ACS patients (before and 2 weeks after the treatment) or 18 healthy subjects were separated and the flow cytometry was used to measure the percentage of Treg. The inhibitory ability of Treg on effector T cells was determined by mixed lymphocyte reaction (MLR). ELISA was used to measure the serum levels of cytokines (IL-10, TGF-β1 and IFN-γ) before and after treatment. The results showed that as compared with normal control group, Treg percentage was decreased significantly (P<0.01), the inhibitory ability of Treg on the T lymphocytes proliferation was reduced (P<0.01), IFN-γ levels were increased and IL-10 and TGF-β1 levels were lowered in ACS patients. After treatment with atorvastatin, Treg percentage and the inhibitory ability of Treg on T lymphocytes proliferation were significantly increased in ACS patients. Serum IFN-γ was decreased significantly, while IL-10 and TGF-β1 were elevated significantly as compared with the non-atorvastatin group. The number of Treg was positively correlated with serum TGF-β1, but negatively with serum IFN-γ and CRP. It was concluded that ACS was associated with decreased number and defected function of Treg, which may play an important role in initiating immune-inflammatory response in ACS. The inhibitory effects of atorvastatin on inflammation in ACS may be due to its beneficial effects on Treg and restoration of immune homeostasis.
Key words: acute coronary syndrome; regulatory $CD4^+CD25^+$ T lymphocytes; cytokine; atorvastatin

The immune-inflammatory response plays pivotal role in acute coronary syndrome (ACS), but the accurate mechanism remains unclear. Many researches have indicated that the general and local immune-inflammatory response in ACS may be caused by activation of immune-inflammatory cells (dendritic cell, monocyte, macrophage and lymphoid cells, etc) and proinflammatory cytokine (IFN-γ) that was abundantly secreted[1, 2]. However, the similar study was absent in the field of inflammatory response in ACS. The recent reports have indicated that, $CD4^+CD25^+$ regulatory T cells (Treg), a fraction of inflammatory regulated negative cells, had important effects to maintain self-immune homeostasis and immune tolerance[3]; Decrease in the number or defected function of the cells can lead to autoimmune disorder (AID). In this study, whether the decrease in the number or defected function of Treg exists in ACS was investigated. Statins have strong anti-inflammatory and immunoregulatory effect[4, 5], and are routinely used for ACS prevention and treatment, but the precise mechanism was still unknown. This study also investigated the possibility of statin drug Atorvastatin for the regulation of immune homeostasis and restoration of Treg functions.

HU Zhenping, female, born in 1966, Doctor in Charge
E-mail: huzhenping@medmail.com
#Corresponding author

1 MATERIALS AND METHODS

1.1 Subjects

Between Jan. to Aug. 2005, 48 cases of ACS (32 males and 16 females with the age of 43—76 years old) were admitted in our hospital and met the WHO standard classification of coronary heart disease (CHD). All cases had at least 50% stenosis of coronary artery inner diameter on coronary arterioangiography. The patients with tumors, infections, autoimmune diseases, severe liver or kidney diseases, hematological disorders and those taking immunosupressants were excluded from this study. Eighteen individuals whose coronary artery was normal were chosen as controls (11 males and 7 females with the age of 38—75 years old).

1.2 Reagents

RPMI1640, 10% fetal bovine serum (FBS) and L-glutamine were from Gibco (USA). Other reagents included MTT and DSMO (Jingmei Biotech Co., Ltd., China); PE-anti-CD25 antibody, FITC-anti-CD4 antibody, FITC-mIgG antibody, PE-mIgG2b antibody (BD, USA). Human interferon-γ (IFN-γ), interleukin-10

(IL-10) and transforming growth factor (TGF-β1) ELISA kit were products of R& D (USA).

1.3 Grouping

Patients with ACS were randomly divided into group C receiving conventional therapy (*n*=24) and group C+A receiving conventional therapy+atorvastatin (Lipitor 10 mg, once daily, *n*=24). Peripheral blood in patients with ACS was collected before and 2 weeks after treatment, and that in the individuals (group N) with normal coronary artery was collected only once upon hospitalization.

1.4 Determination of Treg Percentage and Sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood (10 mL) by standard gradient centrifugation (1000 r/min for 15 min) with lymphocytes separation solution. PBMCs were enriched by removal of the adherent cells and adjusted to a density of 1×10^6/mL. T cells were labeled with FITC-anti-CD 4 and PE-anti-CD 25 for 30 min, washed 3 times with PBS (with 5% FCS) and resuspended in 0.5 mL PBS. $CD4^+CD25^+$ T cells were detected by flow cytometry (Becton Dickinson, USA). $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were also sorted for the following experiments. All the procedures were done at 4 °C.

1.5 Inhibitory Effect of Treg on T Lymphocytes Proliferation

Autologous $CD4^+CD25^-$ effector T cells (2×10^5) were co-cultured with different densities (0.2×10^4, 1×10^5, 2×10^5) of $CD4^+CD25^+$ T cells (37 °C, 5% CO_2) in a 96-well plate supplemented with 10 U/mL IL-2, 0.5 μg/mL the soluble anti-CD3 antibody and allogenic PBMC feeder cells (1×10^5, 3000 rad irradiated). All groups were in triplicate. After culture for 72 h, the supernatant was discarded and then MTT solution was added to the wells at 25 μL/well. After incubation for another 4 h, DMSO (100 μL) was added to each well. Absorbance (*A*) was measured with ELISA reader at dual wavelengths of 540 nm.

1.6 Blood Biochemistry and Measurement of Cytokines

Fasting (12 h) blood samples were collected. Blood sugar and lipids were determined routinely. Highly sensitive CRP was measured by turbidimetry. IFN-γ, TGF-β1 and IL-10 was tested by ELISA.

1.7 Statistical Analysis

Metric data were analyzed by *t*-test, and numeration data were compared between groups by χ^2-test. Linear regression was used to draw the correlation of Treg number and inflammatory cytokines. All the statistical calculations were performed by the SPSS 10.0.

2 RESULTS

2.1 General Conditions

There was no significant difference in the distribution of sex and age among group N, C group and group C+A. No significant difference in the distribution of each biochemical indicator was found between group C and group C+A. Except for atorvastatin, there was no significant difference in the medications as well.

2.2 Percentage of $CD4^+CD25^+$ T Cells in Peripheral Blood

Percentage of Treg/CD4 in peripheral blood of patients with ACS was 2.73%±1.48% before treatment, which was significantly lower than in group N (8.96%±1.71%) (P<0.01). After treatment with atorvastatin for 2 weeks, percentage of Treg/CD4 in group C+A was increased to 6.47%±1.75% (P<0.01), but there was no significant change in group C (3.26%±1.71%). The representative results were shown in fig. 1.

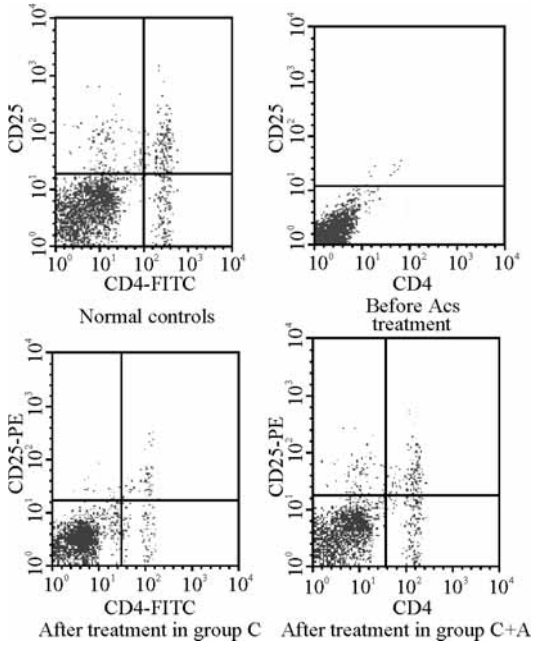

Fig. 1 Flow cytometric analysis of $CD4^+CD25^+$ T cells in peripheral blood

2.3 Inhibitory Effects of Treg in Peripheral Blood on $CD4^+CD25^-$ T Cells

As shown in fig. 2, at the same Treg/effector T-cell ratio, inhibitory ability of Treg on the proliferation of $CD4^+CD25^-$ T cells under stimulation of anti-CD3 antibody in ACS patients was significantly lower than in group N (P<0.01). In group C+A, the inhibitory function of Treg was greatly enhanced as compared with that before treatment (P<0.01) and got close to normal level, but in group C there was no significant change in the Treg suppression function before and after treatment. The inhibitory effects of Treg on the effector T cells were concentration-dependent.

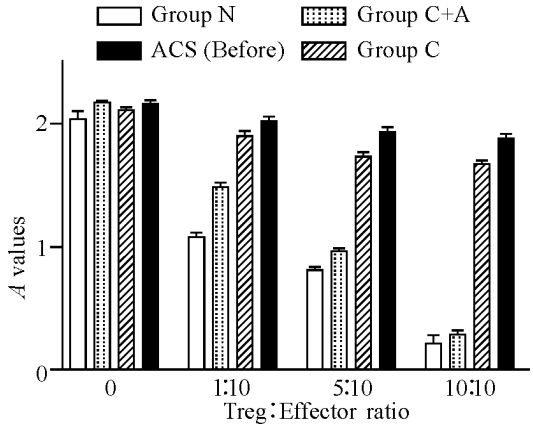

Fig. 2 Comparison of Treg against $CD4^+CD25^-$ T cell proliferation among different groups

2.4 Determination of Cytokines in Circulation

Proinflammatory cytokine (IFN-γ) in circulation in patients with ACS before treatment was higher than in group N, but anti-inflammatory cytokines (IL-10 and TGF-β) were lower than in group N. Before and after treatment there was no significant changes in IFN-γ, IL-10 and TGF-β1 in group C. However, in group C+A, IFN-γ was significantly reduced and IL-10 was significantly elevated. The results were shown in table 1.

Table 1 Cytokines in circulation in different groups (pg/mL)

Groups		IFN-γ	IL-10	TGF-β1
Group C	Before	48.2±9.2*	13.8±3.7*	41.4±8.3*
(*n*=24)	After	37.2±7.1	15.7±7.4	38.6±8.3
Group C+A	Before	47.5±8.5*	14.5±1.6*	40.8±10.2*
(*n*=24)	After	23.2±4.6**	28.6±4.2**	92.7±13.6**
Group N (*n*=18)		13.7±1.9	36.5±7.7	98.1±16.57

*P<0.01 as compared with group N; **P<0.01 as compared with those before treatment

2.5 Change of Biochemical Indicators before and after Treatment

Before treatment, LDL-C (3.0±0.7 mmol/L) in ACS group was significantly higher than in group N (2.1± 0.6 mmol/L, P<0.01). There was no significant difference in LDL-C between group C and group C+A. Before treatment, hsCRP (6.4±1.8 mg/L) in ACS group was significantly higher than in group N (2.5±0.6 mg/L, P<0.01). After treatment with Atorvastatin for 2 weeks, blood hsCRP was significantly decreased to 2.9±1.1 mg/L (P<0.01, as compared with that before treatment).

2.6 Analysis of Correlation

The number of Treg was negatively correlated with circulatory levels of IFN-γ (r= − 0.45, P<0.05) and hsCRP (r= − 0.63, P<0.01), but positively correlated with TGF-β1 (r=0.74, P<0.01).

3 DISCUSSION

The destruction of immune homeostatic mechanism (immunotolerance) can result in autoimmune disorders that attack directly at self-antigen. Though atherosclerosis is not yet categorized as autoimmune disorder, massive evidence demonstrates that the immune-inflammatory response which targets directly at heat-shock protein (HSP) or the oxidated low-density lipoprotein has a pivotal role in the pathogenesis of atherosclerosis. For instance, there are many antigen specific T cells to activate HSP60 in unstabilized plaque and circulation in ACS patients[2]; the onset of ACS induced by chlamydia pneumoniae infection is also associated with the immune response to HSP60[6]. These all indicate that the patients with ACS have the impairment of regulatory mechanism for immune homeostasis.

Recent studies revealed that $CD4^+CD25^+$ Treg play an important role in the development and the maintenance of the immunotolerance[3]. The activation of autoreactive T cells, macrophages and natural killer cells is inhibited by Treg through cell-cell contact or the release of anti-inflammatory cytokines (IL-10 and TGF-β1). Changes in number and(or) function of Treg exist in many autoimmune diseases. Meanwhile, Treg also regulate the immune-inflammatory responses in tumors, transplantations and infections. Mallat *et al* had reported that infusion of Treg to ApoE gene knock-out mice could prevent the development of AS[8]. Through FCM and cell proliferation assays *in vitro*, it was found in this study that the number of circulatory Treg in patients with ACS was significantly reduced as compared with that in normal controls. The number of Treg and inflammatory markers (IFN-γ, hsCRP) in circulation were negative correlated. Further more, the inhibitory function of Treg isolated from ACS patients to auto-T cells was impaired even at the ratio of Treg/ effector of 1:1. These results suggested that Treg might play an important role in inflammatory response of patients with AS.

The mechanism of the decreased number of Treg in ACS is not fully understood. It has been known that the maturity of DCs has a key role in differentiation of T cells[7]. Our previous study already showed that DCs in patients with ACS were highly mature[1], which help T cell to differentiate toward the Th1, and down-regulate the number of circulatory Treg. The differentiation of Treg is also related to the levels of cytokines (IL-10 and TGF-β1). In this study, the levels of IL-10 and TGF-β1 in patients with ACS were lower than in the control group. As Treg can secrete IL-10 and TGF-β1, the causal relation of IL-10, TGF-β1 and Treg is not clear yet. The exact mechanism of Treg function disturbance in patients with ACS is not well understood. Because Treg functions through the way of the cell-cell contact *in vitro*, it is inferred that it may be due to the loss of some cell surface signaling molecules.

Statin drugs have anti-inflammatory and immunoregulatory effects besides lowering lipids and have become the first-line medication to prevent and treat AS and its complications. Previous researches of anti-inflammatory and immune regulatory mechanism about Statins were based on their effects of T-cell proliferation[5]. In this study, it was discovered that after short-term treatment with Atorvastatin, the number of Treg in patients with ACS was obviously elevated and the inhibitory effects on the proliferation of effector T cells were also enhanced. Simultaneously, the circulatory levels of pro-inflammatory cytokines (IFN-γ and CRP) were significantly decreased, while those of the anti-inflammatory cytokines (IL-10 and TGF-β1) were increased greatly. Although Atorvastatin is not a typical immunosuppressant, it can serve as an immunoregulatory agent to help regulate the positive/negative immunity balance and maintain the homeostasis in patients with ACS. The mechanism how Atorvastatin influence Treg is not clear, one of the explanations is that maturation of DC is repressed by Atorvastatin, while immature DC can induce the differentiation of Treg[9]. To further understand the anti-inflammatory and immune regulatory mechanisms of Statins can help to expand its indications beyond cardiology.

REFERENCES

1 Ranjit S, Li D Z, Zeng Q T *et al*. Differentiation of dendritic cells in monocyte cultures isolated from patients with unstable angina pectoris. Int J Cardiol 2004;97(3):

551-556.
2 Li D Z, Ranjit S, Wu W *et al*. Dendritic cell mediated heat-shock protein 60 specific T cytotoxicity in acute coronary syndrome. Chin Ciucu J (Chinese), 2005,5: 340-343
3 Sakaguchi S, Sakaguchi N. Regulatory T cells in immunologic self-tolerance and autoimmune disease. Int Rev Immunol, 2005,24(3-4):211-226
4 Li D Z, Ranjit S, Zeng Q T *et al*. Effects of atorvastatin on the function of dendritic cells in patients with unstable angina pectoris. Chin J Intern Med (Chinese), 2004,6: 429-432
5 Fehr T, Kahlert C, Fierz W *et al*. Statin-induced immunomodulatory effects on human T cells in vivo. Atherosclerosis, 2004,175(1):83-90
6 Hoshilda S, Nishino M, Tanouchi J *et al*. Acute Chlamydia pneumoniae infection with heat shock protein 60-related response in patients with acute coronary syndrome. Atherosclerosis, 2005,183(1):109-112
7 Bacchetta R, Gregori S, Roncarolo M G *et al*. CD4+ regulatory T cells: mechanisms of induction and effector function. Autoimmun Rev, 2005,4(8):491-496
8 Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T cell responses: potential role in the control of atherosclerosis. Curr Opin Lipidol, 2005,16(5):518-524
9 Verginis P, Li H S, Carayanniotis G. Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4+ CD25+ T cells. J Immunol, 2005,174(11):7433-7439

(Received April 24, 2007)