



Changes of circulating CD4⁺CD25⁺CD127^{low} regulatory T cells in patients with acute coronary syndrome and its significance

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ABSTRACT. The aim of this study was to investigate the changes of circulating CD4⁺CD25⁺CD127^{low} regulatory T cells (Treg) in patients with acute coronary syndrome (ACS) and its significance. The experiment was divided into three groups: ACS (48 patients), stable angina pectoris (SAP) (24 patients), and normal controls (24 subjects). The CD4⁺CD25⁺CD127^{low} Treg cell counts were tested by flow cytometry, and the levels of high-sensitivity C-reactive protein (hs-CRP) and peripheral blood leukocytes (PWBCs) were determined in the peripheral blood of each group; comparisons were made among groups. The frequency of CD4⁺CD25⁺CD127^{low} to CD4⁺ cell in the ACS group ($3.18 \pm 1.76\%$) was significantly lower than those observed in control ($5.64 \pm 1.63\%$) and SAP ($5.60 \pm 1.56\%$) groups ($F = 25.247$, $P < 0.01$), while the hs-CRP and PWBC levels in the ACS group were significantly higher than those in the control group ($P < 0.05$). In addition, the reduced frequency of CD4⁺CD25⁺CD127^{low} to CD4⁺ cells was negatively correlated with the increased hs-CRP and PWBC counts by correlation analysis, and the related coefficients (r) were -0.518 and

-0.311, respectively ($P < 0.01$). These findings indicate that the decrease of the frequency of Treg cells in the peripheral blood of patients with ACS might destroy the balance of tolerance of the peripheral immune system and might activate inflammation, thus participating in the occurrence and development of the pathological processes of atherosclerosis.

Key words: Acute coronary syndrome; Regulatory T cells; Flow cytometry; Hypersensitive c-reactive protein; White blood cells

INTRODUCTION

The incidence and mortality of cardiovascular disease have increased over the years as living standards have gradually increased. Acute coronary syndrome (ACS), including unstable angina (UA) and acute myocardial infarction (AMI), are the most common serious cardiovascular illness seen in the clinic; the onset of ACS is sudden, and the disease progresses very quickly and has a high mortality. Atherosclerosis (AS) (Singh et al., 2008) is the primary pathological basis of ACS, which is considered as a chronic inflammatory disease. Immune cells, especially CD4⁺T cells, have been shown to play an important role in ACS. Regulatory T cells (Treg cells) are a subgroup of CD4⁺T cells with special function in immune regulation, and play important roles at all stages of AS and ACS. Research (Ling et al., 2008) has shown that the counts of CD4⁺CD25⁺ Treg cells in the peripheral blood of patients with ACS have decreased in recent years. CD127, a recently identified surface marker for Treg cells, has not been researched to a significant degree in ACS; therefore, in this study we detected the changes of CD4⁺CD25⁺CD127^{low} Treg cells in the peripheral blood in patients with ACS, stable angina pectoris (SAP), and in normal controls by using flow cytometry, and calculated its relationship to the levels of high-sensitivity C-reactive protein (hs-CRP) and peripheral white blood cells (PWBCs) in order to discover the effect of immune adjustment in ACS.

MATERIAL AND METHODS

Patients

We recruited 96 patients who had been hospitalized for coronary artery angiography from August 2012 to June 2013 in the Department of Cardiology of the Second Affiliated Hospital of Anhui Medical University. Patients were classified into three groups according to the diagnosis and classification standards on coronary heart disease issued by the World Health Organization in 1979: 1) the ACS group consisting of 48 patients comprising 18 with AMI and 30 with UA; 2) the SAP group with 24 patients in total, and 3) the control group, in whom other organic heart disease had been ruled out by clinical symptoms, electrocardiogram, chest cardiac color, Doppler echocardiography, and coronary angiography examination; this group included 24 subjects. Exclusion criteria for all patients were tumor, infection, autoimmune disease, severe liver and kidney disease, blood system diseases, and recent use of immune-suppressants.

Main instruments and equipment

Human CD127 single resistance (clone hIL-7R-M21) tagged by phycoerythrin (PE), the

mice single resistance (clone U7.27) labeled by fluorescein isothiocyanate CD25 (FITC), and PE-Cy5-CD4 as a negative control reagent were all purchased from Beckman Coulter (Brea, CA, USA). The hemolysis agent was 0.83% ammonium chloride solution, prepared with distilled water by the Blood Disease Laboratory of the Second Affiliated Hospital of Anhui Medical University. The flow cytometry instrument was the FC500 series from Beckman Coulter, and the wavelengths of fluorescence excitation used for detection were 488 and 633 nm. The CXP system software was used for analysis.

Experimental method

Serum biochemical analysis: all patients were tested for routine blood, liver, and kidney function, blood glucose, and blood lipids using conventional methods, and samples were tested for hs-CRP using an immune scattering turbidimetric method at the same time.

Flow cytometric detection of Treg cell percentages

We obtained 2 mL blood samples from all patients within 12 h after admission. Samples were collected from patients in a horizontal position with strict aseptic techniques, treated with heparin for anti-coagulation, and taken to the epidemiology laboratory and tested within 4 h.

First, we set the window for lymphoid cells according to the forward and lateral dimensions before detection; 10,000 or more cells were sorted in each sample. The whole blood samples were centrifuged at 1500 rpm for 5 min, and then the supernatant was discarded. The precipitated cells were collected and dissociated to generate a cell suspension through the addition of phosphate-buffered saline with centrifugal washing three times; finally the cell number was adjusted to $1-6 \times 10^6/\text{mL}$. A three-color direct immunofluorescence labeling method was used in our experiment: aliquots of 100 μL anticoagulant-treated whole blood were added to two separate tubes; to one was added a total of 10 μL antibody mixture containing CD25-FITC, CD127-PE, and CD4-PC5-Cy5; to the other was added 10 μL antibody mixture including CD4-PC5-Cy5, FITC-IgG1, and PE. After the solutions were incorporated thoroughly, the two tubes were incubated at room temperature for 15 min in the dark, and then 1 mL hemolysis reagent was added and the tubes placed in a water bath at 37°C for 10 min. Immediately after completion of hemolysis, flow cytometry analysis was performed; after testing, the data were stored as Listmode files.

Statistical analyses

All analyses were conducted by the SPSS (Statistical Package for the Social Sciences) 17.0 software (SPSS, Chicago, IL, USA). Data are reported as means \pm SE in the figures. Differences were evaluated using one-way ANOVA for multiple comparisons for measurement data with normal distribution; further comparisons were performed by the least significant difference test. The Pearson χ^2 test or the Fisher exact test was performed for the comparison between groups of measurement data. Analyses of some indicators were conducted using Pearson's straight and correlation analyses.

RESULTS

Basic clinical characteristics

No significant differences were identified in age, gender, smoking status, hypertension, fasting plasma glucose, total cholesterol, three acyl glycerin, low-density lipoprotein cholesterol, or other risk factors of coronary heart disease between the three groups ($P > 0.05$). The high-density lipoprotein cholesterol levels in the ACS and SAP groups were significantly lower than those in the normal control group ($P < 0.01$), while the difference between ACS and SAP groups was not obvious ($P > 0.05$), as shown in Table 1.

Table 1. Comparison of the three groups.

	Normal control group (N = 24)	SAP group (N = 24)	ACS group (N = 48)	F value	P value
Male/female (patients)	15/9	15/9	34/14	0.750	0.687
Hypertension (patients)	9	15	30	4.571	0.102
Diabetes (patients)	9	15	30	0.643	0.725
Smoking (patients)	8	15	18	5.236	0.073
Age (years)	58.63 ± 10.34	56.88 ± 9.09	61.77 ± 10.19	2.137	0.124
Glu (mM)	5.50 ± 0.66	5.73 ± 0.52	5.60 ± 1.18	0.350	0.706
TC (mM)	3.86 ± 0.84	3.99 ± 1.21	3.62 ± 1.06	1.119	0.331
TG (mM)	1.14 ± 0.45	1.09 ± 0.52	0.91 ± 0.60	1.691	0.190
LDL-C (mM)	2.43 ± 0.59	2.32 ± 1.05	2.41 ± 0.87	0.144	0.866
HDL-C (mM)	1.30 ± 0.32	0.84 ± 0.41**	0.91 ± 0.35**	11.894	0.000

**Compared with normal control group, $P < 0.01$. SAP = stable angina pectoris; ACS = acute coronary syndrome; Glu = fasting plasma glucose; TC = total cholesterol; TG = three acyl glycerin; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.

Comparison of Treg cells detected in the peripheral blood of each group

The ratio of CD4⁺CD25⁺CD127 to CD4⁺ cells was significantly lower in the ACS group than in normal control and SAP groups, and the difference was statistically significant ($P < 0.01$); however, the difference between the SAP and normal control groups was not statistically significant ($P > 0.05$); as shown in Table 2 and Figure 1A-C. The ratio of Treg cells to CD4⁺CD25⁺CD127^{high} cells in the ACS group was lower than that in the normal control group ($P < 0.01$), while the difference between the SAP and normal control groups was not statistically significant ($P > 0.05$), as shown in Table 2.

Table 2. Flow cytometric detection of peripheral blood markers in each group (means ± SD).

	Normal control group (N = 24)	SAP group (N = 24)	ACS group (N = 48)	F value	P value
Ratio of CD4 ⁺ CD25 ⁺ CD127 ^{low} cells to CD4 ⁺ cells (%)	5.64 ± 1.63	5.60 ± 1.56	3.18 ± 1.76***	25.247	0.000
Ratio of CD4 ⁺ CD25 ⁺ CD127 ^{high} cells to CD4 ⁺ cells (%)	5.41 ± 2.01	5.40 ± 2.24	5.69 ± 1.81	0.243	0.785
Ratio of Treg cells to CD4 ⁺ CD25 ⁺ CD127 ^{high} cells	1.30 ± 0.60	1.07 ± 0.41	0.94 ± 0.34**	5.644	0.005

** $P < 0.01$ compared with normal control group; *** $P < 0.01$ compared with SAP group; SAP = stable angina pectoris; ACS = acute coronary syndrome; SD = standard deviation.

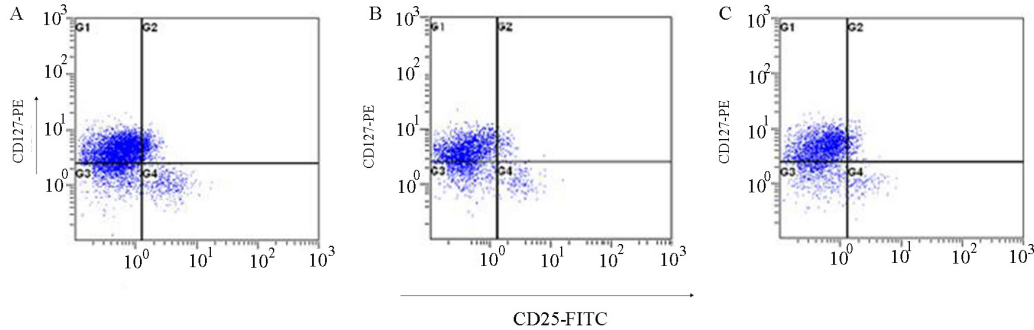


Figure 1. Flow cytometric detection of Treg cells in each group. In each panel, G4: ratio of CD4⁺CD25⁺CD127^{low} to CD4⁺ cells; G2: ratio of CD4⁺CD25⁺CD127^{high} to CD4⁺ cells. PE, phycoerythrin fluorophore; FITC, fluorescein isothiocyanate.

Comparison of the counts of hs-CRP and PWBC in the peripheral blood of each group

According to the results of statistical analysis, the counts of hs-CRP and PWBCs in the ACS group were significantly higher than those in normal control and SAP groups; the difference was statistically significant ($P < 0.05$). The difference of the counts of PWBCs between the SAP and normal control groups was statistically significant ($P < 0.01$), while the difference in hs-CRP between these groups was not statistically significant ($P > 0.05$), as shown in Table 3.

Table 3. Levels of hs-CRP and PWBCs in the peripheral blood of each group (means \pm SD).

	Normal control group (N = 24)	SAP group (N = 24)	ACS group (N = 48)	F value	P value
hs-CRP (mg/L)	2.82 \pm 2.86	3.14 \pm 3.04	7.36 \pm 1.77 ^{###}	39.140	0.000
PWBC (x10 ⁹ /L)	5.54 \pm 1.12	6.86 \pm 0.77 ^{**}	7.65 \pm 1.63 ^{**}	19.696	0.000

^{**} $P < 0.01$ compared with the normal control group; [#] $P < 0.05$; ^{###} $P < 0.01$ compared with the SAP group; hs-CRP = high-sensitivity C-reactive protein; PWBC = peripheral white blood cells; SD = standard deviation; SAP = stable angina pectoris; ACS = acute coronary syndrome.

Correlation of the ratio of CD4⁺CD25⁺CD127^{low} to CD4⁺ cells and the counts of hs-CRP and PWBC

Using straight and Pearson correlation analyses, the results showed that the ratio of CD4⁺CD25⁺CD127^{low} to CD4⁺ cells was negatively correlated to the counts of hs-CRP and PWBC ($P < 0.01$), as shown in Table 4. The hs-CRP and PWBC counts were positively correlated ($r = 0.296$, $P = 0.296$).

Table 4. Correlation between the CD4⁺CD25⁺CD127^{low}/CD4⁺ cell ratio and hs-CRP and PWBC levels.

Items	CD4 ⁺ CD25 ⁺ CD127 ^{low} /CD4 ⁺ cells (%)	
	R value	P value
hs-CRP (mg/L)	-0.518	0.000
PWBCs (x10 ⁹ /L)	-0.311	0.002

hs-CRP = high-sensitivity C-reactive protein; PWBCs = peripheral white blood cells.

DISCUSSION

ACS results from unstable plaques in the coronary arteries that burst, and intraplaque neovasculature, which together contribute to secondary thrombosis and the eventual clogging of the coronary artery lumen. ACS is included among the category of clinical syndromes involving inflammatory and immune reactions (Hansson and Hermansson, 2011). Treg cells are a subgroup of T cells that regulate immune function. CD4⁺CD25⁺ Treg cells, as a minor yet important group of T cells, mediate immune suppression and immune incompetency, and play a role in negative immune regulation and maintain the stability of the internal environment of an organism through contacts between cells and cytokine secretion (Shigematsu et al., 2012). CD127, as the alpha-chain receptor of IL-7, has been shown to play an important role in the proliferation, differentiation, and maturation of T cells. CD127 can effectively remove the effect of T lymphocyte in CD25⁺T cells when used in combination with CD25 high expression and CD127 expression, the result was that can accurately reflect the number of Treg cells (Wang et al., 2008).

Studies (Li et al., 2012; Zhong et al., 2012) have shown that the infusion or induction of Treg cells can delay the development of AS and inhibit the formation of atherosclerotic plaques, which suggested that Treg cells play an important role in the regulation of AS and ACS. Sardella et al. (2007) reported that the number of CD4⁺CD25⁺ Treg cells in patients with ACS is reduced, due to the weakened ability of inhibiting proliferation of CD4⁺CD25⁺ effector T lymphocytes. This study tested the expression level of CD4⁺CD25⁺CD127^{low} Treg cells by flow cytometry in ACS, SAP, and normal control groups. The results showed that the ratio of CD4⁺CD25⁺CD127^{low} Treg to CD4⁺ T cells in the ACS group was significantly lower than those observed in SAP and normal control groups, so we inferred that the lower number of CD4⁺CD25⁺CD127^{low} Treg cells reduced the inhibitory effect on the patient inflammatory response; as a result, the patient was more prone to inflammation and immune response, with the eventual emergence of inflammatory activation and unstable plaques. Therefore, the detection of CD4⁺CD25⁺CD127^{low} Treg cells might be an indicator that could be used to measure the stability of atherosclerotic plaques. However, no difference was found in the expression of Treg cells between the SAP and normal control groups; possible reasons for this might be that the plaques in these patients were stable and the local inflammatory response was less in patients with SAP.

CD4⁺ effector T cells showed high expression of CD127 (CD127^{high}) after activation; however, the true Treg cells showed lower expression of CD127 (CD127^{low}). Therefore, it was suggested that CD127^{high} could be used as a surface marker of activated T cells (Qian et al., 2013). Wang et al. (2009) found that CD4⁺CD25⁺CD127^{low} Treg cells had the ability to inhibit the proliferation of effector T lymphocytes, and with fewer CD4⁺CD25⁺CD127^{low} Treg cells, the proliferation index of CD4⁺CD25⁺CD127^{high} Treg cells increased gradually. Based on the mutual inhibitory effects of Treg cells and T lymphocytes on each other, the control of the frequency of CD4⁺CD25⁺CD127^{high} cells and improvement in the inhibitory effect of T lymphocyte on Treg cells might provide a new immune treatment for patients with ACS (Yu and Cao, 2010).

CRP is one of the most sensitive markers of nonspecific inflammation. The role of hs-CRP has become increasingly apparent in the development of AS, and was considered to be an independent factor that could be used to predict coronary heart disease (CHD) events (Anand and Yusuf, 2010). The results of our study showed that the hs-CRP level increased in normal control, SAP, and ACS groups, and increased significantly in the ACS group, which suggested that environmental disorder, increased inflammatory reaction, stronger immune activity, etc., might exist in the patient.

Hillis et al. (2001) found that the PWBC count level was significantly increased in patients

with CHD, and was more obvious in those with pathological changes in the severe or acute phase, thereby demonstrating that the PWBC count level was significantly positively related to the occurrence of adverse events in patients with ACS in hospital. The results of our study showed that the PWBC count level was significantly increased in both the ACS and SAP groups compared with that in the normal control group. In addition, the frequency of CD4⁺CD25⁺CD127^{low} to CD4⁺ cells was negatively correlated with the hs-CRP and PWBC counts by correlation analysis, which indicated that the inflammatory reaction was highly activated in patients with CHD, and that the strong immune activity was connected to the reduction of CD4⁺CD25⁺CD127^{low} Treg cells; however, the specific mechanism underlying this association is unclear, and need further research.

In conclusion, our study suggested that CD4⁺CD25⁺CD127^{low} Treg cells play an important role in the occurrence and development of AS and ACS. However, due to the small sample size in our study, the experimental results had certain limitations. The immune regulation mechanism of Treg cells in ACS needs future clinical trial research with a larger sample numbers in order to justify the development of new strategies of immunotherapy based on our results.

Conflicts of interest

The authors declare no conflict of interest.

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