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Clinical Study

Evaluation of CD25+CD4+ Regulatory T-Lymphocyte Subpopulations in Coronary Artery Diseases Patients

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Background. The development of atherosclerosis may be associated with a deficiency in the regulatory T-cells, which should serve a protective function and inhibit the accumulation of lymphocytes and macrophages. The aim of this study was the analysis of the T-lymphocyte subpopulations, particularly CD4+CD25+ regulatory T-cells in patients with different form of coronary artery disease. *Materials and Methods*. In the study 30 patients with stable coronary heart disease and 30 patients with unstable coronary heart disease take part. Lymphocytes subpopulations were measured with flow cytometry technique. The analysis of the treated cells parameters was performed with the use of CellQuest program. *Results*. We have observed statistically significant increase in activated lymphocytes subpopulations in patients with unstable coronary artery disease in comparison to stable group and significant decrease in CD25+, CD25/CD3+, and CD25/CD4+ subpopulations in unstable patients comparing to stable patients group. *Conclusions*. A strong interest in regulatory lymphocytes is due to their possible therapeutic use as a factor in modifying the immune response in various diseases. Questions regarding the role of regulatory T-cells in the development of atherosclerosis remain unclear. Mechanisms of the regulatory T-cells impact on suppression of atherosclerosis need more experiments to be done.

1. Introduction

Atherosclerosis is a multifactorial process, which includes interactions between endothelial cells, macrophages, muscle cells, and lymphocytes. The recognition of atherosclerosis as a chronic inflammatory disease contributed to undertaking the research, in which a lot of systemic inflammatory response markers were evaluated for their usefulness in the atherosclerosis progression diagnosis and detecting high-risk acute coronary events. The importance of CRP, fibrinogen, and IL-6 is best known. Other potential factors are the total number of white blood cell count (WBC), the concentration of serum amyloid-A, interleukin, D-dimer, tissue plasminogen activator-1, TNF-α, and pregnancy-associated plasma protein-A (PAPP-A) [1]. The immune response in atherosclerosis begins with the presentation of antigen T-lymphocytes, helper, which induce further immune response involving T-cells and B-cells. Fragments of oxidized low density lipoprotein oxLDL are the major antigens stimulating the organism to the development of inflammatory reaction. Yet we know that some subpopulations of T-lymphocytes lead to increased inflammatory response (Th1) and others to its inhibition (Th2). Imbalance of pro- and anti-inflammatory markers is the first sign stimulating the development of atherosclerotic plaque [2]. It is clear that one of the main components of atherosclerotic plaquesis T-cells. Recent studies have shown that regulatory T-lymphocytes may play a very important role in the progression of atherosclerosis. The development of atherosclerosis may be associated with a deficiency in the regulatory T-cells, which should serve a protective function and inhibit the accumulation of lymphocytes and macrophages [3, 4].

Lipid-laden plaques are covered with a fibrous outer layer, which is in equilibrium with the production of collagen and

its degradation. T-cells in unstable atherosclerotic plaques secrete interferon γ, which inhibits the synthesis of collagen through smooth muscle cells. The combination of reduced collagen synthesis and its accelerated degradation leads to a reduction of the thickness of the fibrous surface layer and then to cracks and fractures [5, 6].

2. Materials and Methods

2.1. Patients. Study group consisted of 30 patients with stable coronary artery disease (group 1) and 30 patients with unstable coronary artery disease (group 2).

The average age of patients with stable coronary artery disease was 55 ± 7.8 years and of patients with unstable coronary artery disease 57 ± 9.4 years. All patients were admitted to the hospital for assessment of angina chest pain. In all patients' cases, angiography was performed to confirm the coronary artery disease. Stable coronary artery disease was defined as typical exertional chest pain relieved by rest or nitroglycerin administration and a positive exertional EKG test. All unstable patients had a stenocardia episode at rest or at minimal exercise which included diagnostic ST segment changes, T wave, and negative cardiac enzymes during the last 48 h before blood sampling.

The criterion for exclusion from the study was the presence of acute or chronic liver disease, kidney disease, cancer, or coexisting autoimmune diseases.

All patients were treated in the Department of Cardiology, Zabrze, Medical University of Silesia in Katowice.

2.2. Flow Cytometry. Evaluation of lymphocyte subsets in peripheral blood was made using standard techniques for immunofluorescence labelling of whole blood. Whole blood was incubated for 30 minutes with the appropriate monoclonal antibodies conjugated with fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), and PerCp-em, and then for 10 minutes with the liquid lysis FACS Lysis (Becton Dickinson) in order to remove erythrocytes. After rinsing the labelled cells twice in PBS, they were introduced into the flow cytometer FAC-Scan (Becton Dickinson), registering 10000 flow cells. For calibration of flow cytometer, to a set of parameters (FSC scatter radius running straight) and SSC (side scattering) calibrator CaliBRITE TM3 was used (Becton Dickinson).

The analysis of the treated cells' parameters was performed with the use of CellQuest program. Marking was carried out in the Silesian Centre for Immunology Lab Pediatrics in Zabrze, certified by European Quality Control Program, Heidelberg (Germany).

We evaluated T-lymphocytes (CD3+), helper T-lymphocytes (CD4+), cytotoxic T-lymphocytes (CD8+), activated T-lymphocytes (CD3+ HLA-DR+), NK natural killer cells (CD16/CD56), the ratio of CD4+ to CD8+ cells (CD4+/CD8+), and subpopulations of CD25+, CD25+/CD3+, CD25+/CD4+, and B-lymphocytes.

2.3. Lipids. Blood samples were collected on EDTA. Plasma was centrifuged at 1500 g for 10 minutes at 4 degrees C and stored at −75 degrees until it was assayed. Total cholesterol, HDL-cholesterol, and triglycerides were determined using a standard set of Alpha Diagnostics Company (Warsaw, Poland), and LDL cholesterol was determined using sets of bioMerieux (France).

2.4. Statistics. The results have been presented as arithmetic mean and standard deviation. In order to compare the groups, Student's t-test was used. As statistically significant, the level of significance $P < 0.05$ was assumed. Statistical analysis was performed using the computer program STATISTICA 7.0.

3. Results

In Table 1 there are results of lipids parameters in patients with stable (group 1) and unstable coronary disease (group 2). We have observed statistically significant increase in total cholesterol and triglycerides concentration in unstable patients comparing to stable coronary disease patients. Statistical analysis of lymphocytes subpopulations in studied group is shown in Table 2. There was observed statistically significant increase in activated T-lymphocytes subpopulation concentration in unstable patients comparing to stable coronary artery group. Information about CD25+, CD25+/CD3+, and CD25+/CD4+ subpopulations is shown in Table 3 and Figure 1. We have observed statistically significant decrease of CD25+, CD25+/CD3+, and CD25+/CD4+ subpopulations in unstable coronary artery disease patients comparing to stable coronary artery disease group.

4. Discussion

For the first time the subpopulation of T-lymphocytes having the ability to inhibit the function of other immune cells was described in the early seventies by Gershon. The breakthrough, which allowed for a closer characterization of these cells, was studied by Sakaguchi et al., who in 1995 discovered that the cells responsible for inhibiting the development of autoimmunity in mice are T-helper lymphocytes, which have receptor to the alpha chain of IL-2 (CD25) on their surface. Phenotypically similar subset of CD4 CD25 has also been shown in humans, as it is present in peripheral blood, thymus, spleen, tonsils, and umbilical cord blood. These populations of CD4+CD25+ T-cells are called regulatory T-cells [7]. They not only are able to inhibit proliferation and cytokine secretion by CD4+CD25−, but they also show suppression effect in relation to CD8 T-cells, NK cells, and dendritic cells. The basic mechanism of suppressive action of CD4 CD25 is based on the direct impact on the target cell [8]. After stimulation of T-cell receptor (TCR) CD4 CD25 the mechanism of active suppression inhibits the activation and the proliferation of CD4 and CD8 cells. There is also a possibility of an indirect suppression action influence on antigen presenting cells (APCs) or through the release of CD4 CD25 suppressor cytokines IL-10. The most important surface molecules involved in active suppression are GITR, CTLA-4, and TGF-beta associated with cell membrane.

Table 1: Lipids parameters in patients with stable and unstable coronary diseases.

	Group 1	Group 2	P value
Total cholesterol mmol/L	4,9 ± 1,1	5,9 ± 1,5	<0,04
Cholesterol HDL mmol/L	1,3 ± 0,3	1,3 ± 0,2	NS
Cholesterol LDL mmol/L	4,1 ± 0,8	4,4 ± 1,1	NS
Triglycerides mmol/L	1,4 ± 0,4	2,1 ± 0,6	<0,03

Table 2: T-lymphocyte subpopulations in patients with stable and unstable coronary diseases.

	Group 1	Group 2	P value
Leukocytes (G/L)	7,12 ± 1,7	6,8 ± 2,7	NS
Lymphocytes (G/L)	2,76 ± 0,29	2,63 ± 0,48	NS
T-lymphocytes (G/L)	1,68 ± 0,42	1,69 ± 0,38	NS
B-lymphocytes (G/L)	0,26 ± 0,06	0,33 ± 0,08	NS
Natural killers (G/L)	0,36 ± 0,1	0,32 ± 0,09	NS
T-helperlymphocytes (G/L)	1,16 ± 0,4	1,00 ± 03	NS
T-suppressor lymphocytes (G/L)	0,7 ± 0,06	0,8 ± 0,07	NS
Activated T-lymphocytes (G/L)	0,23 ± 0,08	0,28 ± 0,02	<0,05

Table 3: CD25+ lymphocytes in patients with stable and unstable coronary diseases.

	Group 1	Group 2	P value
CD25+ (G/L)	0,31 ± 0,04	0,17 ± 0,07	<0,05
CD25+/CD3+ (G/L)	0,18 ± 0,04	0,12 ± 0,02	<0,05
CD25+/CD4+ (G/L)	0,14 ± 0,06	0,10 ± 0,04	<0,05

Recent studies have emphasized the role of transcription factor Foxp3, whose expression is specific for CD4+CD25+ cells and necessary for their proper differentiation [9, 10].

Up to now we have known a few mechanisms regulating the immune response by regulatory lymphocytes [11]. First, it is an active inhibition of T-cells effector through cell-cell interaction. In vitro research demonstrated that IL-10 and TGF-β secreting, associated with cell membrane of T-lymphocytes, is responsible for the inhibition of T-lymphocytes [12, 13]. Nonstimulated regulatory T-cells on their surface show high expression of the receptor alpha chain of IL-2 (CD25) for gamma chain of IL-2 (CD132), and for other molecules, such as HLA-DR, CD45RO, intracellular

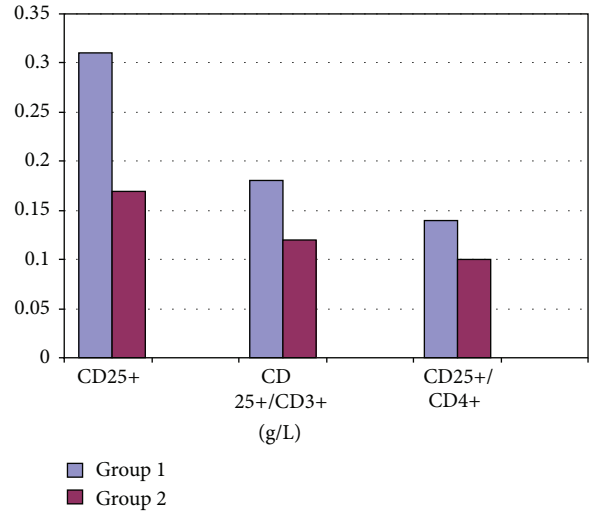

Figure 1

receptor CTLA-4, and CD134 [14]. One of few morphological visible changes occurring during activation of CD4+CD25+ by TCR receptor is the increase in expression of CTLA-4 receptor, which is displaced from the cytoplasm to the cell surface. CTLA-4 ligands protein is B7 receptor on target cells [15, 16]. The result of protein CTLA-4 and B7 receptor connection is a decline in the production of IL-2 by lymphocytes directly exposed to regulatory T-cells. In the aftermath of a reduction in the release of IL-2, a decrease of proliferation of other lymphocytes follows [17]. Another factor, which may contribute to the suppressive regulatory CD4+CD25+ T cells function, is GITR receptor. In vitro studies after the use of monoclonal antibodies blocking the GITR receptor showed the weakness of inhibitory properties of regulatory T-cells.

It is unclear how such a small population of cells is able to supervise the other cells of the immune system. Some explanation might be a two-stage theory of regulatory T-cells, called infectious tolerance. This theory divides the mechanism into two events, occurring sequentially one after the other [18, 19].

In the first stage, CD4+CD25+ cells in direct contact with CD4+CD25− T-cells induce their anergy releasing by them IL-10 and TGF-beta. In this way suppressor cells are formed, which are able to secrete IL-10 and TGF-beta. In the second stage, pregenerated suppressor cells inhibit subsequent CD4+CD25− [20].

CD4+CD25+ subpopulation specializes in the suppression of Th1 cells. Regulatory T-cells deficiency results in the development of various inflammatory diseases, including atherosclerosis.

Some experimental studies have reported that the lack of regulatory T-cells accelerates the development of atherosclerosis in experimental animal models [21]. Regulatory T-cells are responsible for the destabilization of atherosclerotic plaques. Their deficiency contributes to plaque rupture, which entails further consequences and leads to platelet activation, their adherence and aggregation, formation of

thrombin, fibrinogen and fibrin deposition, and localized thrombus formation. Aggregates of platelets and thrombus by coronary artery embolization can cause regional myocardial ischemia or infarction [22]. Various reports inform of the decrease in lymphocytes regulatory T-cells concentration in peripheral blood of patients with type 1 diabetes, rheumatic diseases, or other immunological diseases [21, 23]. Our results indicate a statistically significant decrease in the total concentration of CD25 cells in patients with unstable coronary artery disease compared to the patients with stable coronary artery disease. Statistically significant was also a decrease in the percentage of lymphocytes CD25+/CD3+ and CD25+/CD4+ in the group with unstable coronary artery disease compared with patients with stable coronary artery disease. We have also observed a statistically significant increase in the percentage of activated T-lymphocytes in patients with unstable coronary artery disease compared with patients with stable coronary artery disease. Mor et al. also showed decreased amount and activity of regulatory T-cells in patients with coronary artery disease [9].

Boer et al. conducted one of the few experiments on humans. By means of immunohistochemistry, they assessed the presence of regulatory T-cells in atherosclerotic plaques taken during surgery. They showed that the number of regulatory T-cells in atherosclerotic lesions was lower than in normal tissues. This may indirectly prove the participation of these cells in the formation of atherosclerotic plaques in humans [24].

5. Conclusion

A strong interest in regulatory lymphocytes is due to their possible therapeutic use as a factor in modifying the immune response in various diseases [25]. Many questions regarding the role of regulatory T-cells and their role in the development of atherosclerosis remain unanswered. Mechanisms of the regulatory T-cells impact on suppression of atherosclerosis remain unclear, whether the impact is dependent on the production of cytokines or cell-cell contact or both. We do not know where this regulation takes place [26, 27]. We hope that future study explains the role of regulatory T-cells in the development of atherosclerosis [11, 12, 14, 21, 28].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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