Level of Regulatory T-lymphocytes (CD4+CD25+FoxP3+) in Patients with Unstable Angina

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The role of regulatory T cells (Treg - cells) in the development of atherosclerotic lesions of the vascular wall is the subject of the leading laboratories. Treg - cells are found in atherosclerotic plaques of carotid and coronary arteries of a human. The study included men (n = 16) with a mean age of 61,5 ± 9,3 years, admitted to the intensive care unit with a diagnosis of unstable angina, and conditionally healthy men (n = 8), matched by age. A comparative analysis showed that in patients with unstable angina, there was a significant increase in Treg- cells (CD4+ CD25 + FoxP3+), and the downward trend in T-helper cells (CD4+). Perhaps, this increase in Treg- cells suppresses the further activation of atherosclerosis by inhibiting the proliferation and activation of T-helper cells. Besides, negative correlation between Treg-cells (CD4 + CD25 + FoxP3 +) and T-helper cells (CD4+), and the positive relationship between Treg-cells (CD4 + CD25 + FoxP3 +) and LDL-C are established.

Keywords: T-regulatory lymphocytes, unstable angina, atherosclerosis.

Introduction. During last decades, study of T regulatory cell-mediated suppression is in the focus of immunological research. First they have been characterized by S.Sakaguchi et al, [11] as CD4+CD25+ T cells in study of auto-immune diseases of mice. Regulatory T cells are supposed to play a significant role in maintaining of the immune homeostasis. They are able to suppress activation, proliferation and affective functions of a wide range of immunocompetent cells, including CD4+ and CD8+ T-cells, natural killers (NK) and naturally killing T (NKT)- cells, B – cells and antigen presenting cells *in vitro* and *in vivo* [15, 9].

The intracellular transcription factor FOXP3 is a specific marker of Treg cells. Expression of this protein defines the ability of regulatory T cells to inhibit promotive part of pro-inflammatory cytokine genes [8, 13]. Regulatory Tcells play a key role in the immune system due to their unique ability to control the immune response, they predict autoimmune diseases, allergy, and reaction of transplant rejection, maintain nutritional and transplant tolerance. This role have first been identified in mice in which a deficiency or removal of Treg cells have resulted in development of

autoimmune gastritis, thyroiditis, diabetes, and intestinal inflammatory disease. Further studies revealed the defects in CD4+CD25+FOXP3+ Treg cells to facilitate development of autoimmunity and the processes to be cancelled by transfer of Treg cells. However, regulatory Tcells can also play a negative role in body. Thus, FOXP3+ Treg cells suppress antitumural immunity, thus facilitating tumult progress [17, 9].

The role of Treg cells in development of atherosclerotic lesion of vascular walls is the subject of research of many leading laboratories. Treg cells have been identified in atherosclerotic plaques of human carotid [18] and coronary arteries [5,3]. A significant role of regulatory T lymphocytes in suppression of atherogenesis and stabilization of atherosclerotic lesions have been demonstrated on models of atherosclerosis of mice [14, 19]. Such an effect of regulatory T lymphocytes is considered to be predetermined by suppressive activity in reference to "proatherogenically" effective T lymphocytes, suppression of differentiation of O-type T helpers from 1-and 2-type T-helpers, as well as production of anti-inflammatory cytokines by regulatory T-lymphocytes. The immunoinflammatory response is considered to play a significant role in the course of ischemic heart diseases. Specifically, an inflammatory destructive process characterized by considerable infiltration of monocytes \ macrophages and T-lymphocytes secreting proinflammotory cytokines is of importance in development of an unstable atherosclerotic plaque [12, 2].

Materials and research methods. The study covers 16 patients, aged 46-72 (an average age being 61,5 ± 9,3), admitted to the intensive care unit of the municipal enterprise "Yakutsk City Clinical Hospital" diagnosed with unstable stenocardia. Nine conditionally healthy males comparable by age and not having a history of the ischemic heart disease and vascular pathology were included into the control group. Acute inflammatory, auto immune and oncological diseases made the criteria of exclusion from the study. The study was performed with the consent of patients and according to the ethic norms of the Declaration of Helsinki (2000 г.). A comparative group was made of 8 practically healthy males comparable by age.

Venous blood sampling (7-8 ml) was taken in the morning on an empty stomach during first three days after admission of the patient to the intensive care and resuscitation unit. Mononuclear cells of the periphery blood (MNC-PB) have been separated by means of phycol-verographine density-gradient centrifugation of heparinized venous blood. The mononuclear cell fraction has been washed with phosphate-salt buffer (PSB) and re-suspended, and the cell concentration has been determined. Cells were selected in the quantity of 1 x 10^6 and precipitated with centrifugation, supernatant was removed and re-suspended in 100 mkl buffer solution.

To define Treg cells through assessment of FoxP3 transcription factor expression, FoxP3 Staining Kit-PE («BD Pharmingen»™) was used, the study was conducted according to the protocol presented by the company-producer. The quantity of $1x10^6$ cells in 100 mkl PSB was transported into 12,75 mm test tube («Falcon», «BD Bioscience»), 20 mkl of CD4 - FITC and CD25 - APC antibodies were added to the suspension of cells, were incubated for 20 min in the dark at the indoor temperature and 2 ml of PSB were washed, and the sedimentation was re-suspended in the residual volume of PSB. The cells were fixed during 10 min in 2 ml of the corresponding reagent (included in the kit) in the dark at the room temperature and 2 ml of PSB were washed two times. Then, 0,5 ml of the permeabilitating solution included in the kit was added to the sedimentation, was incubated during 30 min at the indoor temperature, 2 ml of PSB were washed and the sedimentation was re-suspended in 100 mkl of PSB. 20 mkl of FOXP3 antibodies were added to the cell suspension, were incubated for 30 min in the dark, the cells were washed in 2 ml of PSB two times, 200 mkl of PSB were added and examined in the running cyto-fluorimeter FACSCantoII («BD Bioscience») using the software FACSDiva («BD Immunocytometry Systems»). Immunophenotype cells characterized by morphological features corresponding with those of helper T lymphocytes defined by the character of side light scattering (SSC) and expression of CD4+ were selected for processing the obtained data at the first stage of the analysis. The content of T regulatory cells was defined as a share of CD25+ FoxP3+ immunophenotype cells. The statistical analysis was held using «SPSS 17.0 for Windows». The equality of selected average values was corrected according to the Student parametric T criteria and Mann-Whitney non-parametric U criteria for independent samples. To define the tightness of links between the quantitative data under study, a correlated analysis with calculation of coefficients and the Spirman rank correlation was conducted. Variations at $p<0,05$ were taken as statistically relevant.

Results and discussion. The comparative analysis of the relative content of T lymphocytes revealed a decrease of the mean values of total T lymphocytes (CD3+) and T-helpers (CD4+) in patients with unstable stenocardia as compared with those of the control group, and they fall within the range of reference values. It is worthwhile to note here that the content of T helpers (CD4+) in the group of patients evidences the tendency towards a decrease ($p=0,063$) as compared with that of the control group (45,50 ± 1,55 %) (Table).

A significant variation has been identified in the relative content of regulatory CD4+CD25+FoxP3+ T lymphocytes, and the expression of these cells in patients with unstable stenocardia is increased 1,71 times than that in the control group ($p=0,039$) (Fig.) These data indirectly confirm our results concerning the relative deficit of T cell immunity obtained earlier [1],

as well as the data referring to an increase of the anti inflammatory IL- 10 in patients with unstable stenocardia as compared with that of patients with stable stenocardia [4]. The regulatory T lymphocytes are supposed to have an anti- inflammatory effect and are able to stabilize the atherosclerotic process [10].

Table 1

Content of lymphocytes in peripheral blood, %

Rates	Patients with unstable stenocardia (n=16)	Control group (n=8)	p ...
Total T-cells CD3+	66,25 ± 3,34	69,50 ± 4,13	
T-helpers CD4+	39,43 ± 2,50	45,50 ± 1,55	0,064
Treg-cells CD4+CD25+Foxp3+	5,56 ± 0,54	3,25 ± 0,25	0,039

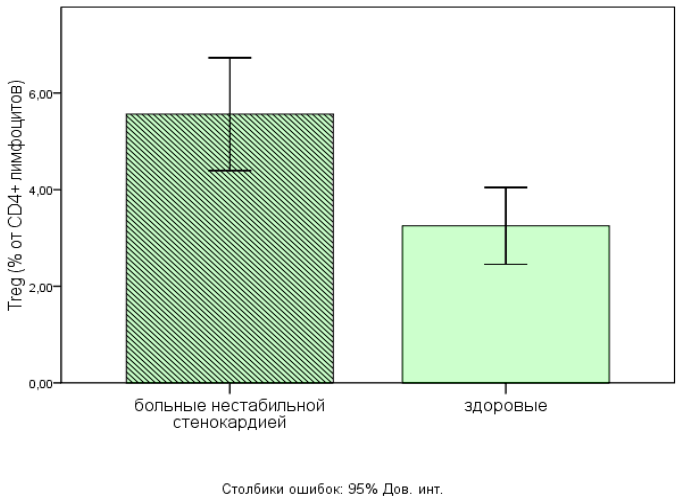

Fig. Content of regulatory T-lymphocites (CD25+CD4+Foxp3+)

Currently, the following subpopulations of regulatory T-cells: Tr1, type 1 regulatory T cells, Th3, type 3 helper T cells, CD8+ iTreg induced regulatory T cells and CD4+CD25+FOXP3+ Treg – cells have been identified. Tr1 cells are formed in the periphery, without the thymus gland, thus they belong in adaptive and/or inducible regulatory T cells (iTreg). iTreg cells are induced under the stimulation with activating agents (specifically, antigen) and are formed in the course of the

immune response. Differentiation of iTreg is antigen-dependent and is performed under certain conditions: in the presence of cytokines with immunomodulating properties and sensitive to these cytokines APC. Tr1 cells mostly secret IL-10, and TGF and IL-5 in small quantities. They are able to inhibit functions of Th1 and Th2 either *in vitro*, or *in vivo*. Tr1 cells control development of autoimmune processes, regulate activation of naïve cells and Tcells of memory, functions of dendrite cells (DC) and development of the immune response to various pathogens, allotypic antigens, and participate in the process of tumult growth [7, 6]. Suppressive properties of Tr1 cells are associated mostly with the ability of IL-10 secretion, as their functions can be disturbed by use of antiIL-10 monoclonal antibodies (мАТ) [6].

We have performed the correlated analysis that has identified the following associations of Treg cells: the negative link with T helpers (CD4+) and (r= - 0,523; p=0,018), and rather strong positive correlative associations with the level of the total cholesterol (r=0,702; p=0,004), atherogenic ХС-ЛПНП (r=0,724; p=0,002) and γ-glutomiltransferase (r=0,661; p=0,005).

Thus, a significant increase of the level of Treg cells in the serum of patients with unstable stenocardia allows us to hypothesize that it is this increase of Treg cells that inhibits further activation of the atherosclerotic process by inhibition of T helper proliferation and activation, notably, by means of increased secretion of anti inflammatory IL-10 cytokine, thus securing more favorable prognosis for the disease (the patients under study were discharged in better condition). The obtained data require further study.

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