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Research article

The severity of internal carotid artery stenosis is associated with the circulating Th17 level

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ABSTRACT

Aim: Immune and inflammatory reactions contribute to the progression of atherosclerosis. The walls of the different arteries and segments of the arteries have heterogeneous haemodynamic and histological features. We aimed to explore the relationship between the circulating T-cell subsets and the abundance of carotid atherosclerosis in different segments of carotid arteries.

Methods: 70 patients underwent ultrasound duplex scanning to determine the degree of stenosis of the common carotid artery (CCA), the CCA bifurcation or the internal carotid artery (ICA). The blood frequencies of T-, B-, NK-cells, regulatory T cells (Treg), activated T-helpers (Th), IL10-producing Th, Th1 and Th17, as well as blood levels of hsCRP, sCD25, IL10 and IL17a were assessed.

Results: The frequencies of Th17 were increased in patients with ICA stenosis >35% and >50% vs. patients with ICA stenosis <35%. Th17 blood level ≥0.55 % of lymphocytes was associated with more severe stenosis of ICA (OR 4.3 (1.0–17.6), $p < 0.05$ for ICA stenosis of 35–50% and 6.8 (1.3–35.0), $p < 0.05$ for ICA stenosis >50%). BMI positively correlated with the CCA bifurcation stenosis degree ($r = 0.33$, $p < 0.05$).

Conclusion: The severity of ICA stenosis can be associated with the circulating Th17 level.

1. Introduction

Atherosclerosis (AS) is currently considered as a chronic inflammatory process in the arterial wall due to the accumulation of lipoproteins and their derivates with antigenic properties. These molecules are phagocytosed and presented to T-lymphocytes by dendritic cells and macrophages inducing T-cell activation and proliferation. T-lymphocytes modulate activation of macrophages and other cells in the vascular wall and regulate the inflammatory process [1, 2].

The largest data on regulatory and effector T-cell blood content and function in atherosclerosis was obtained in patients with ischaemic heart disease. A decrease in circulating regulatory T-cells (Treg), accompanied by an elevation in circulating T-helpers (Th) 17, has been shown in patients with severe atherosclerotic lesions of the coronary arteries [3, 4]. Similar changes in the immunological balance,circulating Th17 increase and Treg reduction, were observed in patients with severe carotid artery lesions or unstable plaques [5, 6, 7]. It has also been demonstrated that Treg are able to pass the blood brain barrier after ischemia, playing a key

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role in promoting ischemic brain damage [8]. Thus, a significant increase of Treg content has been described in patients with acute cerebrovascular symptoms and critical carotid artery stenosis [9]. We have previously demonstrated a decrease in Treg/Th17 ratio in patients with carotid atherosclerotic plaques (AP) > 30% [10]. The prognostic significance of reduced IL-10-producing CD4+ T-cell blood frequencies for coronary atherosclerosis progression was observed [11]. Also in critical carotid artery stenosis, an increase of circulating IL-10-producing CD4+ T-cells has been demonstrated, suggesting that such Treg subpopulation can exert a protective role against aortic wall rupture [12].

The walls of the different arteries and segments of the arteries have heterogeneous haemodynamic and histological features. Previously, Dalager S. et al [13] indicated that the initiation, the progression rate, and the types of AP are artery-related. Since extracranial carotid arteries include segments with different histological types, we proposed that the Treg/Th17 balance may differentially contribute to the atherosclerotic process in different segments of the carotid arteries. In the present study, we analysed the relationship between T-cell subset frequencies in blood and the abundance of carotid atherosclerosis in the distal segments of the common carotid artery (CCA), the CCA bifurcation and the internal carotid artery (ICA).

2. Material and methods

This study was approved by the Institutional Ethics Committee. Written consent was obtained from each patient. 70 males with different severity of carotid atherosclerosis were enrolled. The exclusion criteria were acute coronary syndrome or interventions in the previous 6 months, the history of stroke, neoplasms, liver or renal failure, infectious/inflammatory disease, decompensated diabetes mellitus and current use of immunosuppressive drugs. None of the patients had stenosis of the vertebral arteries>20% or severe bilateral stenoses. All patients enrolled in this study had been receiving statins for at least 1 month before the enrollment.

Vascular ultrasound. Duplex scanning of the carotid arteries was performed using a high-resolution ultrasound system with linear array transducer 3–9 MHz (PHILIPS iU22 ultrasound system, Philips Inc., Eindhoven, the Netherlands). B-panel of scanning, color-flow mapping in energetic and velocity panels, Doppler frequency shift spectrum analysis were used. Real-time images of CCA/ICA were synchronized with R-wave in the ECG. AP were assessed in the distal parts of the CCA, in the CCA bifurcation, in the ICA bilaterally in longitudinal (anterior, lateral and posterior plane) and transversal views. Carotid plaques were defined as focal structures encroaching into the arterial lumen of at least 0.5 mm or 50% of surrounding intima-media thickness value, or demonstrating thickness greater than 1.5 mm as measured from the intima-lumen interface to the media-adventitia in compliance with the Mannheim carotid intima-media thickness and plaque consensus (2011) [14]. The severity of carotid artery stenosis was determined by the ECST criteria (baseline stenosis site artery diameter/stenosis site artery diameter x 100 %) [15]. The plaques were considered unstable if heterogeneous plaque structure was accompanied by irregular plaque surface including ulcerations (>2mm) and/or hypoechoic component [16].

Lymphocyte immunophenotyping. Whole blood was collected in a sodium citrate anticoagulated vacutainer tube. The samples were processed within 2 h after being collected. For surface antigen staining the following antibodies and reagents were used: CD3-FITC, CD3-PerCP, CD4-FITC, CD19-FITC, CD8-PE, CD(16+56)-PE, CD25-PE, CD127-PC5, CD45-APC, and lysing and fixing solutions (Beckman Coulter, Becton Dickinson Immunocytometry Systems, eBioscience). The intracellular antigens analysis was performed in mononuclear leukocytes. The cells were isolated by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich). For cytokine detection, mononuclear cells were additionally cultivated in the presence of 25 ng/ml PMA, 1 μg/ml ionomycin, and 10 μg/ml monensin for 4 h. Cell staining was performed with CD4-PC5, FoxP3-Alexa488, interleukin (IL)10-PE, INFγ-PE, IL17a-Alexa488 and appropriate isotypic controls, and with a FoxP3 Staining Buffer Set (all reagents from eBioscience) in accordance with the manufacturer's manual. The samples were analysed with two-laser FACS Calibur flow cytometer equipped with CellQuest Pro software (Becton Dickinson Immunocytometry Systems). Lymphocytes were identified by light scattering parameters and CD45 expression pattern. The blood frequencies of CD3+CD4+, CD3+CD8+ T cells, CD19+ B cells, CD3-(CD16+56)+ NK cells were estimated. Treg were identified as CD4+CD25highCD127low and CD4+FoxP3+, activated T-helper cells (Th-act) as CD4+CD25lowCD127high, IL10-producing T cells as CD4+IL10+, Th1 as CD4+INFγ+, Th17 as CD4+IL17a+ cells.

Cytokine analysis. The sCD25 and IL10 levels in serum were measured by chemiluminescence with an Immulite 1000 (DPC-Siemens) analyzer according to the manufacturer's manual. IL17a plasma level was assayed by ELISA (Bender Medsystems). The hsCRP concentration was detected in serum by nephelometry (Behring nephelometer, Marburg GmbH).

Statistics. The data are presented as median (25^{th}-75^{th} percentile). Kruskall-Wallis ANOVA and Mann-Whitney U tests were used in multiple or paired comparisons, respectively. Fisher's exact two-tailed test was used in paired comparisons of binary features. Spearman's test was used for correlation analysis. Odds ratio (OR) calculation were performed using MedCalc. The differences were considered statistically significant at $p < 0.05$.

3. Results

According to the vascular ultrasound data we determined four degrees of the carotid stenosis severity for each localization of the atherosclerotic lesions: ≤ 25%, >25 ≤ 35%, >35 ≤ 50% and >50% in CCA, CCA bifurcation and ICA. The distribution of the patients according to the severity of stenosis and localization of the lesion is shown in Figure 1. The unstable plaques were predominantly detected in ICA (16%) or bifurcation of CCA (20%) rather than in CCA (1%).

The main clinical characteristics of the patients and the estimated immunological parameters according to the severity of ICA stenosis are presented in Table 1.

Patients with the ICA stenosis of >35% or >50% had increased Th17 blood frequencies compared with patients with the ICA stenosis of less than 35% ($p < 0.05$ and $p = 0.05$, respectively). The frequencies of Th17 ≥ 0.55 % were associated with more severe stenosis of ICA (OR 4.3 (1.0–17.6), $p < 0.05$ for ICA stenosis >35 ≤ 50% and 6.8 (1.3–35.0), $p < 0.05$ for ICA stenosis >50%). The proportion of unstable plaques increased in accordance with the increase of the degree of ICA stenosis, however, there were no significant differences in the Th17 blood levels between the groups of patients with unstable and stable lesions in all studied localizations of carotid arteries as well as between patients with different severity of the ICA stenosis.

The CCA or CCA bifurcation stenosis above 50% were lacking (Figure 1) and the analysis was performed in the groups of patients with the degree of stenosis of ≤25%, >25 ≤ 35% and >35%. The groups of patients with different severity of CCA atherosclerotic lesions or CCA bifurcation lesions were comparable in basic clinical characteristics, biochemical and immunological parameters (data not shown). Arterial hypertension was more commonly observed in patients with CCA stenosis of >25 ≤ 35% (91%) and >35% (100%) compared with patients with CCA stenosis ≤25% (38%) ($p < 0.05$). Body mass index (BMI) showed a slight positive correlation with the CCA bifurcation stenosis degree ($r = 0.33$, $p < 0.05$), but not with CCA or ICA stenoses.

No differences in circulating CD4+CD25highCD127low, CD4+Foxp3+ Treg, IL10-producing T-cells, Th-act or Th1, CD4+, CD8+ T cells, B cells, NK cells as well as in hsCRP, sCD25, IL10 or IL17a blood levels were detected in patient groups with different localization of carotid AS.

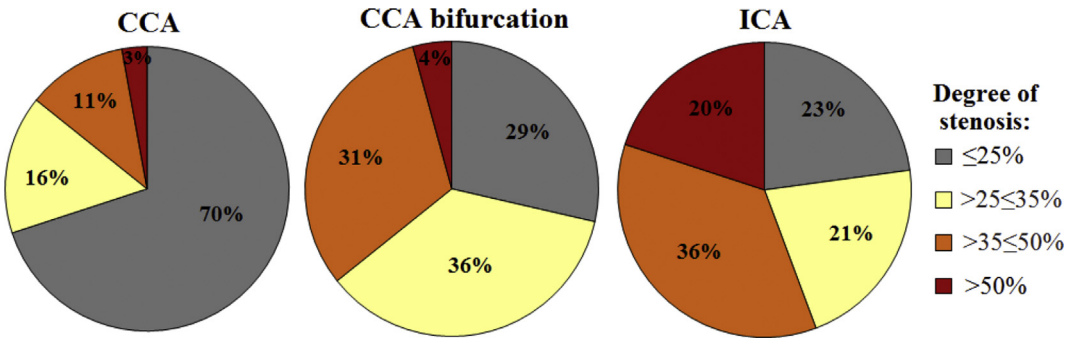

Figure 1. The proportion of patients with different severity of carotid stenoses at each localization.

Table 1. Clinical characteristics and immunological parameters in patients with different severity of ICA stenosis.

	Division into groups according to the degree of stenosis of the ICA (n = 70)				*p*
	≤25% n = 16	>25 ≤ 35% n = 15	>35 ≤ 50% n = 25	>50% n = 14	
Age, years	61.0 (54.0; 73.0)	65.0 (51.0; 74.0)	63.0 (56.0; 67.0)	58.0 (54.0; 65.0)	*0.72*
Smoking, n (%)	5 (31%)	8 (53%)	12 (48%)	7 (50%)	*0.61*
Body mass index, kg/m2	28.0 (25.0; 34.0)	26.0 (25.0; 29.0)	29.0 (27.0; 33.0)	29.0 (26.0; 30.0)	*0.20*
Arterial hypertension, n (%)	11 (73%)	10 (66%)	19 (76%)	9 (64%)	*0.88*
Previous myocardial infarction, n (%)	4 (25%)	4 (26%)	10 (40%)	4 (28%)	*0.79*
Diabetes mellitus, n (%)	3 (18%)	1 (7%)	2 (8%)	0 (0%)	*0.61*
Unstable plaques, %	6%	7%	8%	35%	*0.09*
Total cholesterol, mmol/l	4.3 (3.9; 4.8)	4.6 (3.5; 4.8)	4.8 (3.9; 5.4)	4.6 (3.5; 6.0)	*0.45*
Triglycerides, mmol/l	1.3 (1.0; 1.7)	1.3 (0.9; 1.7)	1.4 (1.0; 2.2)	1.5 (1.2; 1.9)	*0.67*
HDL, mmol/l	1.1 (1.0; 1.1)	0.8 (0.8; 1.3)	0.9 (0.8; 1.2)	1.1 (0.8; 1.2)	*0.75*
LDL, mmol/l	2.6 (2.0; 2.8)	2.4 (1.9; 3.1)	2.9 (1.7; 3.6)	2.8 (2.2; 4.9)	*0.63*
Glucose, mmol/l	5.5 (5.1; 6.0)	5.3 (4.6; 5.7)	5.3 (4.9; 6.4)	5.7 (5.0; 6.6)	*0.78*
hsCRP, mg/l	1.9 (0.6; 2.0)	2.6 (0.8; 7.8)	3.2 (0.8; 6.4)	3.1 (0.8; 5 .6)	*0.66*
sCD25, U/ml	564.0 (487.0; 1081.0)	851.0 (650.0; 1080.0)	828.0 (458.0; 1205.0)	615.0 (556.0; 1047.0)	*0.92*
IL10, pg/ml	2.5 (1.4; 3.0)	2.5 (1.7; 3.2)	2.3 (1.6; 3.4)	2.2 (1.7; 2.8)	*0.94*
IL17, pg/ml	1.0 (0.2; 1.5)	1.1 (1.0; 3.6)	2.5 (1.0; 6.0)	1.2 (0.5; 3.5)	*0.13*
Leukocytes, mln/ml	7.2 (6.7; 7.9)	7.7 (6.5; 8.5)	7.0 (6.4; 8.4)	7.9 (6.7; 9.4)	*0.55*
Lymphocytes, mln/ml	24.0 (18.0; 29.0)	25.0 (21.0; 31.0)	25.0 (21.0; 30.0)	28.0 (22.0; 31.0)	*0.26*
CD4+ T-cells, % lym	39.0 (35.5; 45.0)	43.0 (34.5; 52.0)	40.0 (35.0; 43.0)	42.5 (34.0; 48.0)	*0.63*
CD4+CD25lowCD127high Th-act, % lym	17.5 (12.3; 22.4)	16.5 (11.9; 20.5)	17.4 (11.8; 23.2)	16,8 (9,0; 20,3)	*0.85*
CD4+CD25highCD127low Treg, % lym	1.9 (1.2; 2.5)	2.0 (1.5; 2.8)	2.0 (1.5; 2.5)	1,7 (1,3; 2,1)	*0.54*
CD4+Foxp3+ Treg, % lym	3.2 (2.6; 3.9)	3.3 (2.7; 4.2)	2.9 (2.5; 3.6)	3,3 (2,0; 4,0)	*0.75*
CD4+IL10+ T-cells, % lym	1.2 (0.9; 1.9)	1.1 (0.9; 1.7)	1.5 (1.1; 1.9)	1,5 (0,8; 2,3)	*0.70*
Th17, % lym	0.46 (0.34; 0.70)	0.45 (0.33; 0.66)	0.57 (0.40; 0.85)	0.87 (0.28; 1.50)	*p2vs.3 0.04 p2vs.4 0.05*
Th1, % lym	9.0 (8.0; 13.0)	9.9 (6.5; 13.0)	8.1 (6.1; 11.1)	8.5 (7.7; 10.0)	*0.48*

Data are presented as median and interquartile range. P–the significance level for the comparison of four groups of patients; if the statistical significant differences were observed during the four group comparison, the pairwise comparison was performed and p for pairwise comparison is presented.

4. Discussion

Atherosclerosis is a systemic disease affecting various arterial regions. The aorta and the large arteries (carotid, coronary, less often renal or mesenteric arteries) are the most common localizations of AP. Both common AS risk factors, e.g. BMI, dyslipidemia, hyperglycemia, and recently discovered factors (immunological imbalance), develop systemically. To date, it is unclear why various vascular pools are affected with AS unequally [17].

T-lymphocytes regulate the inflammatory process in the vascular wall. Th1 are the main subpopulation of effector T-cells in AP. Th1 exert a pro-atherogenic activity secreting the pro-inflammatory cytokines INFγ and tumor necrosis factor [18, 19]. Treg antagonize effector cells via producing the anti-inflammatory cytokines, such as transforming growth factor-β and IL10, thus exhibiting anti-inflammatory and anti-atherogenic activity [20, 21, 22, 23, 24]. The contribution of Th17 to the development of AS is ambiguous. Some studies demonstrate the pro-inflammatory and pro-atherogenic role of Th17 and IL17 [7, 25, 26], while others show Th17 promote plaque fibrosis and stabilize AP [27].

The initiation, progression rate, and phenotypes of atherosclerotic plaques are artery-related. Clarification of the reasons for these differences is important in prevention and treatment of atherosclerosis and its complications. The haemodynamic and histological features of the arterial wall in different segments of brachiocephalic arteries may influence AS development. The ICA is a distal part of the CCA with a smaller diameter. The blood flow in the CCA bifurcation and the ICA is turbulent. The CCA belongs to the arteries of elastic type, while the proximal segment of ICA is a muscular-elastic type [28]. The immune

inflammation is accompanied by the accumulation of mononuclear leukocytes, proliferation and cell migration, which may occur to a greater extent in muscular arteries, such as the coronary arteries or the distal segments of the carotid arteries. Dalager S. et al [13], indicated different histological types of AP in various vascular pools. Intimal thickening without the accumulation of foam cells was more common in the coronary arteries, in contrast to the proximal segments of carotid arteries, where the accumulation of foam cells and AP with a lipid core was detected. There were similarities between the histological types of AP in the distal segments of the carotid arteries and in the coronary arteries.

In the present study, we assessed the relation between the balance of effector and regulatory subpopulations of T-cells and the severity of carotid AS depending on the location of the atherosclerotic lesions. We observed that the circulating Th17 level was associated with accelerated ICA stenosis. Previously, we have demonstrated the association of increased Th17 blood frequencies or decreased Treg/Th17 ratio with an accelerated rate of carotid arteries AS progression [29]. We did not observe any correlation between Th17 or any other T-cell subpopulation blood level and the severity of CCA or CCA bifurcation AS. The role of conventional AS risk factors, such as arterial hypertension or BMI, was more crucial for CCA or CCA bifurcation AS.

By regulating immune responses and modulating tissue inflammation, Th17 cells are now considered as key players in autoimmune reactions [30]. Th17 cells were shown to be implicated in the pathogenesis of psoriasis, rheumatoid arthritis and systemic lupus erythematosus [31, 32, 33]. According to epidemiological data, patients with these so-called Th17-associated disorders exhibit an increased cardiovascular risk and are at risk of adverse outcomes in the case of cardiovascular events [34]. The morphological features of the vascular wall and the haemodynamic factors impact the migration of immune cells into plaques. We suggest immune mechanisms may have ambiguous contribution to the development of AS in different types of arteries. The elevated frequencies of circulating Th17 may be one of the manifestations of the systemic “pro-inflammatory state" and provoke atherogenesis in ICA and bifurcation of CCA.

In the present study we did not observe the differences in the blood levels of IL-10, IL-17 or hsCRP, probably because of definite atherosclerosis in all groups of patients and the small study group.

We hypothesise the immune inflammatory component of atherosclerosis can be artery-related. Further studies are needed to identify the contribution of immune cells to AS progression in various vascular pools. The study was approved by Russian Ministry of Health, project № AAAA-A18-118031390106-1.

5. Limitations

This was a pilot study with a small group of patients enrolled. Only male patients were enrolled, as the cellular immunity parameters vary according to the gender [35, 36, 37]. The extent of atherosclerosis in any other vascular basin except for extracranial segments of brachiocephalic arteries, was not analyzed.

Declarations

Author contribution statement

A. Filatova, A. Potekhina, E. Pylaeva and T. Arefieva: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Osokina: Performed the experiments; Analyzed and interpreted the data.

N. Ruleva: Performed the experiments.

O. Pogorelova and T. Balakhonova: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

M. Tripoten and V. Masenko: Performed the experiments; Contributed reagents, materials, analysis tools or data.

E. Noeva: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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