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$CD4^+CD28^-$ lymphocytes and ischaemic stroke. Part I: $CD4^+CD28^-$ lymphocytes and common carotid artery intima-media thickness

Limfocyty $CD4^+$ $CD28^-$ a udar niedokrwienny mózgu. Część I: Limfocyty $CD4^+$ $CD28^-$ a kompleks błona środkowa–błona wewnętrzna

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Abstract

Background and purpose: More and more data point to the involvement of the $CD4^+CD28^-$ lymphocyte subpopulation in the pathogenesis of ischaemic stroke. This paper attempts to answer the question of whether an increase in the percentage of $CD4^+CD28^-$ lymphocytes in the blood may be associated with carotid artery intima-media thickness (IMT).
Material and methods: The study involved a group of 109 patients, aged 45 to 65 years, including 42 patients with first-ever ischaemic stroke, experiencing symptoms resulting from disturbances of the anterior area of cerebral circulation, arterial hypertension and/or type 2 diabetes mellitus (group 1). Group 2 consisted of 34 patients with above-mentioned risk factors but without ischaemic stroke. The control group comprised 33 healthy individuals. Distribution of sex and mean age was comparable. The IMT of carotid arteries was measured by ultrasonography. Flow cytometry was applied to determine the percentage of $CD4^+CD28^-$ lymphocytes in the peripheral blood.
Results: The IMT was significantly greater in patients with stroke than in patients without stroke. No significant correlation was found between the proportion of $CD4^+CD28^-$ lymphocytes in the blood and the IMT of carotid arteries.

Streszczenie

Wstęp i cel pracy: Coraz więcej danych wskazuje na udział subpopulacji limfocytów $CD4^+CD28^-$ w patogenezie udaru niedokrwiennego mózgu. W pracy podjęto próbę odpowiedzi na pytanie, czy wzrost odsetka limfocytów $CD4^+CD28^-$ we krwi wiąże się z pogrubieniem kompleksu błona środkowa–błona wewnętrzna (KIM) tętnic szyjnych.
Materiał i metody: Do badania zakwalifikowano 109 osób w wieku od 45 do 65 lat, w tym 42 chorych na pierwszy w życiu udar niedokrwienny mózgu, z objawami klinicznymi wynikającymi z zaburzeń przedniego obszaru krążenia mózgowego, nadciśnieniem tętniczym i/lub cukrzycą typu 2 (grupa 1.). Grupę 2. stanowiło 34 chorych z wymienionymi czynnikami ryzyka, ale bez udaru mózgu, a grupa kontrolna (grupa 3.) składała się z 33 osób uznanych za zdrowe. Rozkład płci i średnia wieku były porównywalne. Zaawansowanie miażdżycy określono, mierząc grubość KIM przy użyciu ultrasonografii. Odsetek limfocytów $CD4^+CD28^-$ we krwi obwodowej analizowano za pomocą cytometrii przepływowej.
Wyniki: Odsetek limfocytów $CD4^+CD28^-$ i wartości KIM były znamiennie większe u chorych na udar mózgu. Nie udało się wykazać korelacji pomiędzy grubością KIM tętnic szyjnych a odsetkiem limfocytów $CD4^+CD28^-$ we krwi obwodowej.

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Conclusions: The significant proportion of $CD4^+CD28^-$ lymphocytes in patients with ischaemic stroke points to the involvement of the cells in the pathogenesis of stroke. The $CD4^+CD28^-$ lymphocytes are not involved in the pathomechanism of common carotid arteries IMT thickening in this group of patients.

Key words: ischaemic stroke, intima-media thickness, common carotid artery, ultrasonography, $CD4^+CD28^-$ lymphocytes.

Wnioski: Duży odsetek limfocytów $CD4^+CD28^-$ u chorych na udar niedokrwienny mózgu wskazuje na udział tych komórek w patogenezie udaru. Limfocyty $CD4^+CD28^-$ nie są zaangażowane w patomechanizm pogrubienia KIM tętnic szyjnych wspólnych w tej grupie chorych.

Słowa kluczowe: udar niedokrwienny mózgu, kompleks błona środkowa–błona wewnętrzna, ultrasonografia, limfocyty $CD4^+CD28^-$.

Introduction

It has been proven that carotid artery intima-media thickness (IMT) is an independent risk factor in cerebral infarction [1-8]. Atherosclerosis is a progressive disease whose pathogenesis involves numerous factors, including inflammatory processes. Experimental research has established that apart from macrophages, $CD4^+$ lymphocytes are also activated in the inflammatory process. As a result of activation the lymphocytes proliferate, thus intensifying the inflammatory response occurring within the vascular wall [9-12]. In the pathogenesis of unstable coronary disease an important role is played by a subpopulation of those lymphocytes, devoid of the CD28 receptor ($CD4^+CD28^-$) [13]. The lack of CD28 receptor expression on the surface of T lymphocytes is caused by a block in the transcription of the receptor protein coding gene [14]. The cells may release pro-inflammatory cytokines, especially interferon γ, producing destructive metalloproteinases, perforins and granzyme B [15-17]. $CD4^+CD28^-$ lymphocytes are described as typically oligoclonal cells, resistant to apoptosis and not subject to systemic control. They are a result of long-lasting antigenic stimulation [17]. More and more data point to the involvement of the $CD4^+CD28^-$ lymphocyte subpopulation in the pathogenesis of ischaemic stroke [18,19]. We hypothesize that $CD4^+CD28^-$ lymphocytes are associated with IMT.

This paper attempts to answer the question of whether an increase in the proportion of $CD4^+CD28^-$ lymphocytes in the blood may be associated with carotid artery IMT thickening.

Material and methods

One-hundred and nine patients (56 males and 53 females), aged 45 to 65 years, were qualified for the study and were divided into three groups. Group 1 comprised 42 patients (23 males and 19 females), aged 56.5 ± 5.8 years, treated at the Pomeranian Medical University Neurological Department, with their first-ever ischaemic stroke, experiencing clinical symptoms resulting from disturbances of the anterior part of the cerebral circulation, arterial hypertension and/or type 2 diabetes mellitus (the most important and independent risk factors for atherosclerosis) and who underwent different examinations, including ultrasonography examination, in the first 24 hours of their disease. Group 2 was made up of 34 persons (17 males, 17 females), aged 55.6 ± 6.1 years, recruited from patients of the Department of Endocrinology, Arterial Hypertension and Metabolic Diseases as well as employees of the Neurological Department and their family members, with arterial hypertension and/or type 2 diabetes with no clinical symptoms of a previous or current stroke. Group 3 (control) comprised 33 persons (16 males, 17 females), aged 55.5 ± 4.3 years, who had not undergone cerebrovascular episodes and were not suffering from arterial hypertension or type 2 diabetes. Distribution of sex and mean age was comparable in all three groups.

Exclusion criteria were applied to all three groups and included: (1) recent infection, chronic immunological and inflammatory diseases (multiple sclerosis, Graves-Basedow disease, Hashimoto disease, connective tissue systemic diseases, hepatic cirrhosis, viral hepatitis, Crohn disease, ulcerative colitis), (2) proliferative diseases of the hematopoietic system or other neoplastic diseases, (3) cardiovascular diseases carrying a risk of hemodynamic or embolic cerebral infarction (atrial fibrillation, acute coronary episodes, including myocardial infarction, sick sinus syndrome), (4) peripheral arterial disease (past or present upon admission to hospital).

The study was approved by the Bioethics Committee of Pomeranian Medical University, and all recruited participants gave their informed consent.

Patients' history, medical documentation and administered drugs constituted the basis for the diagnosis of arterial hypertension and type 2 diabetes. Arterial blood pressure was measured twice at the hospital, following a 10-minute rest in a sitting position, at the beginning and

at the end of the visit. Newly detected hypertension was diagnosed when during both measurements systolic blood pressure (SBP) equalled ≥ 140 mm Hg and/or diastolic blood pressure (DBP) was ≥ 90 mm Hg. The participants were divided into three subgroups, according to the values of their arterial pressure, in compliance with the classification of the European Society of Hypertension: grade 1 hypertension patients (SBP ≥ 140-159 mm Hg and/or DBP ≥ 90-99 mm Hg), grade 2 hypertension patients (SBP ≥ 160-179 mm Hg and/or DBP ≥ 100-109 mm Hg) and grade 3 hypertension patients (SBP ≥ 180 mm Hg and/or DBP ≥ 110 mm Hg) [20].

Newly detected diabetes was diagnosed when the fasting blood glucose level upon two measurements equalled or was higher than 126 mg/dL or when the blood glucose level upon two measurements, at any time of day and regardless of mealtime, equalled or was higher than 200 mg/dL. An alternative criterion of diabetes diagnosis was blood glucose level ≥ 200 mg/dL two hours after the oral administration of 75 g of glucose (glucose tolerance test). According to the WHO criteria, the following conditions were diagnosed: prediabetes, with impaired fasting glucose (IFG, 100-125 mg/dL) or impaired glucose tolerance (IGT), if blood glucose level was 140-199 mg/dL in the second hour of the glucose tolerance test [21].

Atherogenic dyslipidaemia was diagnosed when plasma low-density lipoprotein (LDL) cholesterol concentration was > 115 mg/dL, high-density lipoprotein (HDL) cholesterol concentration was < 40 mg/dL in males and < 45 mg/dL in females, and when triglyceride concentration was > 150 mg/dL [22].

Inflammatory state was diagnosed when the following conditions occurred in patients: increased serum concentration of C-reactive protein (CRP) (> 5 mg/dL) and/or accelerated erythrocyte sedimentation rate (ESR) (> 15 mm/h) and/or leukocytosis > 10 000/μL with absent infection and/or increased fibrinogen concentration (> 400 mg/dL) (according to the standards of the Central Laboratory of the University Hospital).

A complete set of laboratory examinations, including blood cell count, blood glucose level, CRP, ESR, coagulation tests and lipid profile, was performed directly after the patient's admission to hospital.

In order to rule out myocardial infarction or ischaemic heart disease, electrocardiogram (ECG) and laboratory examinations were performed in group 1 patients (troponin T level and creatine kinase cardiac isoenzyme – CK-MB in serum).

Computed tomography (CT) examination of the brain of group 1 patients was performed during the first few hours of their hospitalization in order to differentiate the type of stroke. CT was performed at the Department of Imaging Diagnostics and Intervention Radiology using the Picker PQ5000 CT scanner; slice thickness of 10 mm was used (lamp parameters: 120 kV and 250 mA, matrix 512 × 512).

Doppler ultrasonography examination of the carotid arteries was performed at the Ultrasonography Laboratory of the Neurological Department with the ESAOTE AU 5 device, equipped with a 7.5 MHz linear probe. Thickness and echogenicity of the vascular wall of common carotid arteries (CCA) were analyzed. The artery was divided into 2 segments in accordance with the Mannheim Intima-Media Thickness Consensus [24].

The first segment included the distal 2 cm of the vascular wall of the CCA, adjacent to the dilatation of the bifurcation. The second segment – the carotid bifurcation – was included in the carotid dilatation up to the flow divider of the internal and external carotid arteries. The examination was performed in longitudinal projection, in two planes, antero-lateral and postero-lateral and sagittal, so as to evaluate the aspect of the vessel and its patency. The CCA posterior wall IMT was measured in B-mode projection. In every visualized plane and in each of the analyzed segments IMT was measured at 5-mm intervals. The median was calculated for all measurements in the left and right CCA. Similarly, the median was calculated for the bifurcations of those arteries. IMT was defined as the distance between the lumen-intima interface and the media-adventitia interface (Fig. 1). The measurements were made in the abovementioned places, regardless of the occurrence or lack of atherosclerotic plaque. The methodology was used in accordance with the adopted procedure [23-25]. The recommendations of the Polish Neurological Society were taken into account. Ultrasound imaging and IMT measurements were assessed by two persons. Intra-observer and inter-observer coefficient variations were respected, according to the Bland-Altman method [26]. Concurrence for intra-observer variability for p-value was $p < 0.0001$. Inter-observer variability for two readers for p-value also was $p < 0.0001$. Acquired data were archived by means of the 'SARO' ultrasound images archiving system.

Determining CD28 receptor expression on the surface of CD4 lymphocytes

In group 1, the material was collected in the first 24 hours of the cerebral infarction and in groups 2 and 3

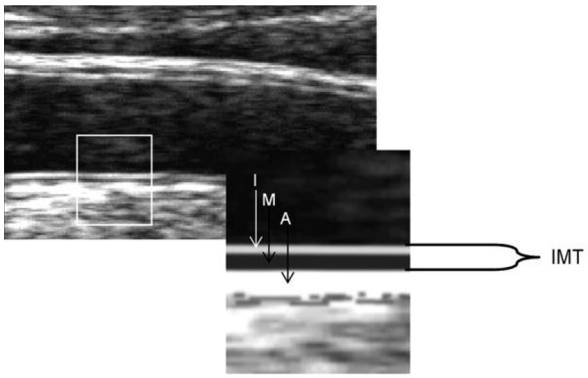

Fig. 1. High resolution ultrasound image of common carotid artery in longitudinal projection. The places of measurements of the distal wall intima-media thickness are marked. On the intermediate lamina the structures of the arterial wall are marked with arrows: I – intima, M – media, A – adventitia

it was collected on the day of their registration for the examination. The expression of CD28 receptor on lymphocytes was evaluated using the FACSCalibur (Becton Dickinson) flow cytometer combined with the sorting device and the Power Mac G4 computer (Cell Quest OS2 software). The number of $CD4^+CD28^-$ lymphocytes was expressed as a proportion of the $CD4^+$ ($CD4^+CD28^-$ and $CD4^+CD28^+$) subpopulation.

The assays were performed at the Pathomorphology Department of the Medical Faculty of Pomeranian Medical University.

Statistical analysis

For measurable variables, the following values were included in the tables: mean, minimal and maximal value as well as standard deviation (SD). For nominal variables, the number and percentage of people demonstrating a given feature within a group were presented. Measurable variables demonstrated distributions that were significantly different from the normal distribution (Shapiro-Wilk test, $p < 0.05$); hence non-parametric tests were employed. In order to demonstrate the significance of the differences among more than two groups, Kruskal-Wallis ANOVA was used, while in order to compare two groups of patients, Mann-Whitney *U*-test was used. In order to compare the measurable interrelated variables (values of parameters measured in the same patient, e.g. on their right and left side), Wilcoxon's pair sequence test was used. The strength of correlation between the variables was evaluated by means of the value of the Spearman rank correlation coefficient (*Rs*). Nominal variables were compared by means of the χ^2 test or the two-tailed Fisher exact test (for 2×2 tables). *P*-value < 0.05 was established as the threshold of statistical significance. Statistical calculations were performed using the Statistica 7.1 program.

Results

Clinical characteristics of the study group ($n = 109$) are shown in Table 1. Significantly more people from group 1 manifested atherogenic dyslipidaemia (83.3% vs. 67.6% in group 2 and 54.5% in group 3; $p < 0.01$ for the comparison between groups 1 and 3). A particularly high percentage of patients in group 1 demonstrated features of inflammatory condition (71.4% vs. 20.6% in group 2 [$p < 0.001$] and 3% in group 3 [$p < 0.001$]). On the other hand, significantly more people categorized in group 2 were treated with antihypertensive drugs ($p < 0.005$) and arterial hypertension lasted longer in their case than in the case of people from group 1 (9 vs. 2.5 years, $p < 0.005$).

The IMT was significantly greater in group 1 pa - tients, compared to group 2. Even greater differences were present between group 1 or 2 and the control group (group 3). No significant differences were found between the IMT of the left and right CCA and their bifurcations. Also no significant difference in the IMT on the side of the stroke and on the opposite side was found.

The age of the patients correlated significantly with the IMT of the examined arteries in patients from group 1 ($Rs = 0.382, p = 0.012$), while the duration of arterial hypertension correlated significantly with the IMT in patients from group 2 ($Rs = 0.510, p = 0.002$).

The proportion of $CD4^+CD28^-$ lymphocytes in the blood was 11.52% (range, 0.8-44.08%) in group 1, 11.35% (0.8-57.8%) in group 2, and 3.37% (0.3-50.81%) in group 3. There was no significant difference between the proportion of examined lymphocytes in groups 1 and 2 ($p = 0.97$). However, in both groups it was significantly higher than in group 3 (group 1 vs. group 3, $p < 0.0001$; group 2 vs. group 3, $p < 0.001$).

No significant correlation was found between the proportion of $CD4^+CD28^-$ lymphocytes in the blood and the IMT of the CCA or their bifurcations (group 1: $Rs = 0.039, p = 0.806$; group 2: $Rs = -0.240, p = 0.171$; group C: $Rs = 0.306; p = 0.082$).

Similarly, no significant correlation was found between the proportion of $CD4^+$ $CD28^-$ lymphocytes in the blood and the duration of arterial hypertension, its grade and the values of SBP and DBP, or between the

Table 1. Clinical characteristics of the study group ($n = 109$)

Clinical characteristics	Patients with ischaemic stroke (Group 1)	Patients with AH and/or DM type 2 (Group 2)	Controls without AH or DM type 2 (Group 3)	*P*-value (Group 1 vs. Group 2)	*P*-value (Group 1 vs. Group 3)	*P*-value (Group 2 vs. Group 3)
Number of subjects	42	34	33	–	–	–
Age [years]; mean (SD)	55.5 (5.8)	55.6 (6.1)	55.5 (4.3)	NS*	NS*	NS*
Gender						
Female; *n* (%)	19 (45.2%)	17 (50%)	17 (51.5%)	NS**	NS**	NS**
Male; *n* (%)	23 (54.8%)	17 (50%)	16 (48.5%)			
BMI [kg/m^2]; median (range)	28.1 (21.2-41.5)	28.5 (19.9-43.7)	24.9 (21.3-37.7)	< 0.001*	< 0.001*	NS*
AH; *n* (%)	42 (100%)	34 (100%)	–	NS*		
AH duration [years]; median (range)	2.5 (0-20)	9.0 (0-40)	–	< 0.005*		
Maximum SBP [mm Hg]; median (range)	180 (140-280)	172.5 (140-250)	125 (100-135)	NS*	< 0.001*	< 0.001*
Maximum DBP [mm Hg]; median (range)	107.5 (70-140)	100 (70-160)	80 (70-89)	NS*	< 0.001*	< 0.001*
DM type 2; *n* (%)	10 (23.8%)	8 (23.5%)	–	NS*	–	–
Duration of DM type 2 [years]; mean (range)	4.0 (0-16)	6.6 (0-25)	–	NS*	–	
Impaired fasting glucose; *n* (%)	10 (23.8%)	12 (35.3%)	–	NS**	< 0.001*	NS**
Dyslipidaemia; *n* (%)	35 (83.3%)	23 (67.6%)	18 (54.5%)	NS**		

AH – arterial hypertension, DM – diabetes mellitus, SD – standard deviation, BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, NS – non-significant
**ANOVA with Kruskal-Wallis test and Mann-Whitney U-test; **χ^2 test and Fisher exact test*

proportion of the examined lymphocytes and the duration of diabetes.

The relationship between inflammatory condition and IMT in groups 1 and 2 is shown in Table 2. In group 1, in patients with an inflammatory condition, IMT of CCA and their bifurcations was significantly greater than in patients with no features of the inflammatory state. No such interdependence was observed in patients from group 2.

Discussion

In the extensive literature concerning stroke and the structure of IMT, greater and greater significance is attached to the inflammatory and immunological mechanisms involved in the pathogenesis of atherosclerosis and nervous tissue ischaemia. Ultrasound examination, employed to evaluate IMT, is a reliable, accurate and reproducible method [1,8,27]. The ARIC (Atherosclerosis Risk in Communities) and Rotterdam studies have demonstrated that it is easiest to evaluate IMT in CCA, more difficult in its bifurcation and most difficult in the internal carotid artery [28, 29]. Taking this fact into consideration, we have focused on the CCA and their bifurcations in our study. The measurements of the distal wall of the CCA are the most reliable when it comes to the prognosis of the occurrence of cerebrovascular and cardiovascular episodes [30]. High compatibility between ultrasound examination and the results of histopathological examinations has been repeatedly confirmed [31]. Hence, in many studies [32, 33], including ours, only the measurements of the CCA distal wall have been provided. In the groups we examined, mean values of IMT in the CCA and their bifurcations were significantly higher in the patients of group 1 than in group 2, and especially in the patients from the control group. This confirms the above-mentioned significance of IMT as a risk factor in ischaemic stroke.

Table 2. Inflammatory state and intima-media thickness (IMT) in common carotid artery (CCA) in groups 1 and 2

Variable	Patients with an inflammatory state	Patients without an inflammatory state	*P*-value*
Group 1 ($n = 42$)			
IMT of the left and right CCA [mm]; mean (SD)	1.06 (0.24)	0.92 (0.17)	< 0.05
IMT of the left and right carotid bifurcation [mm]; mean (SD)	1.33 (0.40)	1.09 (0.24)	< 0.05
Group 2 ($n = 34$)			
IMT of the left and right CCA [mm]; mean (SD)	0.84 (0.06)	0.90 (0.15)	0.531
IMT of the left and right carotid bifurcation [mm]; mean (SD)	1.03 (0.16)	1.08 (0.15)	0.081

**Mann-Whitney U-test*

IMT – intima-media thickness, CCA – common carotid artery

We observed interdependence between age and IMT in the CCA and in the carotid bifurcations in group 1.

When evaluating IMT, we took into account the occurrence of arterial hypertension and type 2 diabetes. These diseases themselves can influence IMT. A correlation between IMT and the duration of arterial hypertension occurred in our patients without cerebral stroke (group 2). This could be accounted for by the considerably longer duration of arterial hypertension in this group of patients. A significantly greater number of those patients, as opposed to the patients with cerebral stroke, took antihypertensive drugs, which might have prevented the occurrence of vascular episodes. The duration of diabetes was similar in the group of patients with and without stroke.

We decided to check how IMT of the CCA and their bifurcations behaves in the patients with an increased proportion of the $CD4^+CD28^-$ lymphocyte subpopulation. From what can be read in the literature, it appears that the patients with a consecutive stroke had higher mean values of $CD4^+CD28^-$ lymphocytes than the patients with their first-ever stroke, which seems to confirm the involvement of those cells in the pathogenesis of stroke [19].

In group 1, IMT was significantly greater and the proportion of those cells was elevated. Significantly greater IMT in the CCA and their bifurcations in inflammatory patients of group 1 and no such interdependence in group 2 may suggest the role of an inflammatory component in CCA IMT thickening. However, we were unable to demonstrate a correlation between IMT and the proportion of $CD4^+CD28^-$ lymphocytes in peripheral blood. The potential role of these cells in CCA IMT thickening remains unclear.

Conclusions

The increase in the proportion of $CD4^+CD28^-$ lymphocytes in peripheral blood is not associated with carotid artery IMT thickening. The potential role of these cells in IMT thickening remains unclear.

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Disclosure

Authors report no conflict of interest.

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