Original article

Folia Neuropathologica

Circulated $CD4^+CD28^-$ lymphocytes rate and their cytotoxicity and morphological parameters of internal carotid artery atheromatous plaques in patients with atherosclerosis-related ischemic stroke

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Folia Neuropathol 2013; 51 (3): 250-260 DOI: 10.5114/fn.2013.37710

Abstract

Background: *The question about the role of $CD4^+CD28^-$ lymphocytes in the pathogenesis of atherosclerosis-related ischemic stroke is still open.*

Material and methods: *The study included patients, who underwent endarterectomy of internal carotid arteries. The group consisted of patients after stroke and ones without previous stroke. The histopathologic examination of obtained plaques has been performed. The percentage of $CD4^+CD28^-$ lymphocytes in peripheral blood and their cytotoxicity has been assessed for each patient.*

Results: *The global percentage of $CD4^+CD28^-$ lymphocytes in blood was higher in the group of patients with plaques where connective tissue fibers consisted significantly of collagen fibers. The cytotoxicity of analysed cells related to less plaques calcification and presence of cholesterol crystals in the plaque. A multifactor regression of dependent variable presented the last relationship above as a only strong.*

Conclusions: *$CD4^+CD28^-$ cytotoxic lymphocytes seem to be involved in carotid atherosclerotic plaques development. Intraplaque cholesterol deposits may contribute to this process.*

Key words: *$CD4^+CD28^-$ lymphocytes, carotid plaques, stroke.*

Introduction

Inflammatory and immunological factors play an important role in ischemic stroke pathogenesis, taking into consideration their involvement in an ischemic lesion development [9,21].

Over the years, research has also demonstrated the large role of aforesaid factors in the process leading to the acute brain ischemia onset, through development and destabilization of atherosclerotic lesions. It seems that $CD4^+CD28^-$ lymphocytes ($CD4^+CD28^-$ Lc) hold a vital position therein. It is a cell population that

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combines classical CD4+ lymphocytes' (helper T cells, Lc Th) characteristics with those of cytotoxic lymphocytes and natural killer cells. Likewise classical Th lymphocytes, they stimulate and control the immunological reaction. However, unlike classical lymphocytes, they participate directly in the reaction, which is expressed by their ability to infiltrate target tissues and possession of cytotoxic properties [15,16,26,31]. CD4+CD28– Lc are resistant to apoptosis, therefore mechanisms induced thereby work incessantly [30]. CD4+CD28– Lc are a source of large quantities of interferon-γ, a potent macrophage activating factor. Macrophage activation and metalloproteinase excretion is an acknowledged mechanism responsible for the destruction of atherosclerotic plaque fibrous elements, which results in the weakening of the plaque structure and an increased susceptibility to ruptures [18,32]. As a source of granzymes and perforins, the described lymphocytes have an ability to damage the target tissues directly. Cytotoxicity of CD4+CD28– Lc towards endothelial and smooth muscle cells in relation to atherosclerotic plaque was established [15].

It is thought that they may directly damage endothelial cells of intraplaque vessels and consequently lead to the formation of effusions inside a plaque, which undoubtedly contribute to destabilization of atherosclerotic lesions [2,22].

Research on patients with coronary arterial diseases indicates that the described cells play a vital role in the process of coronary artery plaque destabilization and acute coronary syndrome occurrence [11-13].

Our own research has shown so far that the aforesaid cells may also participate in the atherosclerosis-related ischaemic stroke [19]. However, the potential role of the cells in ischemic stroke pathogenesis remains unclear. It seems as if we may seek one in the development of atherosclerotic plaques in extracerebral and intracerebral vessels [6].

The aim of the study was to attempt to address the question of the potential role of CD4+CD28– lymphocytes in the pathogenesis of the atherosclerosis-related ischemic stroke, with special reference to the histopathological picture of carotid atherosclerotic plaques.

Material and methods

The study included 91 patients (25 females and 66 males) aged between 44 and 85 (mean age 67.74), who underwent endarterectomy of internal carotid arteries, with the following comorbidities: arterial hypertension, type 2 diabetes, dyslipidemia, obliterative atherosclerosis of the lower limbs, coronary arterial disease (occurring both separately and in combination with others).

Patients with chronic diseases, where inflammatory and immunological factors play their role, were excluded from the study (systemic connective tissue diseases, viral hepatitis, cirrhosis, ulcerative colitis, Crohn's disease, multiple sclerosis, Hashimoto's thyroiditis, Graves' disease, proliferative diseases of the haematopoietic system, other cancers). Another exclusion criterion was a past surgical procedure performed on a patient within six months directly preceding enrolment in the study.

Microscopic evaluation of carotid plaques

Intraoperatively harvested atherosclerotic plaques were fixed in an 8% formalin solution to be split into five parts. The two most peripherally located parts and one central part, including the area affected by the disease the most, were collected for evaluation. The material was then submerged in paraffin, sliced into 3 micrometer-thick fragments and stained with hematoxylin and eosin (H&E) and using the PAS and Gieson's methods.

The following were included in the assessment: inflammatory infiltrations, plaque vascularisation, presence of intraplaque haemorrhage, mural thrombus, thrombus built into the plaque structure, plaque fibrous component, foam cells, lipid core, cholesterol crystals and calcifications.

A plaque with a mural thrombosis, fibrous cap rupture and also containing rich lipid core (> 1/3 of the plaque thickness) and/or intraplaque haemorrhage and/or thrombi built into the plaque structure, massive or widespread inflammatory infiltrations (with > 200 or between 100 and 200 cells, respectively), numerous vessels (> 9 vessel sections in the visual field), numerous foam cells (occupying > 1/3 of the plaque thickness), a fibrous component with a majority of collagen fibres (> 2/3) or one with an equal proportion of collagen and elastin fibres, was considered microscopically *unstable*.

A plaque having no mural thrombi or fibrous cap ruptures but that had intraplaque haemorrhages, intraplaque thrombi or characteristic of at least four out of following features: cholesterol crystals, massive or widespread inflammatory infiltrations, numerous vessels, numerous foam cells, the above

described structure of a fibrous component, was considered *potentially unstable*.

A plaque with no mural thrombosis, no rupture of the fibrous cap, no intraplaque haemorrhages or thrombosis, with minor or large isolated vessels focus (< 6 or between 9 and 6 of the vessel lumen in the visual field), with isolated minor inflammatory infiltrations (< 100 cells in the visual field), scarce foam cells (< 1/3 of the plaque thickness), with neither of the remaining features described above; with no marked disturbance of fibrous element integrity, with a majority of elastin fibres constituting more than 2/3 of the plaque fibrous component, was pronounced as *stable*.

Plaque calcifications were assessed as numerous (> 8 minor calcifications in the visual field or one large focus occupying more than 1/3 of the plaque thickness), a few minor (between 4 and 8), and isolated minor ones (< 4 focuses in the visual field).

The evaluation was performed on the basis of the American Heart Association Guidelines and an analysis of data available through professional literature, with a particular attention paid to data regarding carotid arteries [2,8,23,29].

Plaques in the ipsilateral artery up to an acute cerebral episode (ischemic stroke or TIA) were regarded as symptomatic. Plaques in patients with no stroke or in the contralateral artery up to a vascular episode were treated as asymptomatic. 46 patients were included in the symptomatic plaque group, while the remaining 45 patients were incorporated into the asymptomatic plaque group.

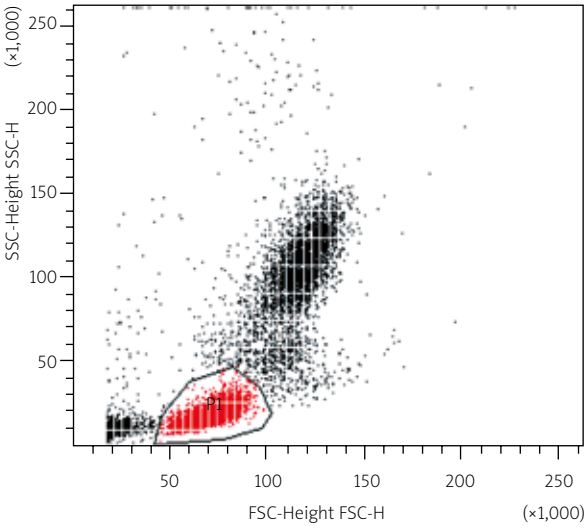

Fig. 1. Gated lymphocyte subpopulation based on SSC vs. FSC measurement.

Flow cytometry

Prior to surgery, each of the subjects included in the study had a 2.7 ml sample of peripheral blood collected to an EDTA tube.

The following antibodies were added to the properly marked tubes (no. 1 and no. 2): to tube no. 1 – control Mouse IgG1-PerCP (Becton Dickinson) and IgG1-PE (Becton Dickinson) antibodies; to tube no. 2 – CD4-PerCP (Becton Dickinson) antibodies and CD28-PE (Becton Dickinson) antibodies. Then, 50 µl of full blood was added to each of the tubes with antibodies. The blood-antibody mix was stored in dark, at room temperature. Post incubation permeabilizing liquid (Dako) was added to samples no. 1 and no. 2. After remixing and incubating, the samples were washed with 5% phosphate buffer saline (PBS). After centrifugation and removing the supernatant, fixing liquid was added to a pellet and to tubes no. 1 and no. 2; the following antibodies were added respectively: Mouse IgG2b-FITC model antibodies (Becton Dickinson) and Anti-human Perforin-FITC (Becton Dickinson). After storing again, washing with a 5% PBS, centrifugation and removing the supernatant, 1% formalin solution was added to a pellet. The expression of the investigated CD28 receptor on lymphocytes and the expression of perforins in the studied cells were evaluated with the application of a FACSCalibur flow cytometer coupled with a sorting device. The analysis was performed on a research computer using Cell Quest OS2 software. Lymphocyte subpopulation was differentiated among lysed whole blood cells, basing on correlated measurements of Forward- and Side-Scattered light (FSC and SSC) (Fig. 1) [21].

The number of the studied $CD4^+CD28^-$ lymphocytes was expressed as a percentage of $CD4^+$ lymphocytes ($CD4^+CD28^-$ and $CD4^+CD28^+$). The cytotoxicity of the studied cells was assessed on the basis of intracellular perforin expression and was presented as a percentage of $CD4^+CD28^-$ lymphocytes ($Perforin^+CD4^+CD28^-$ and $Perforin\text{-}CD4^+CD28^-$) (Fig. 2). Control Mouse IgG1-PerCP (Becton Dickinson) and IgG1-PE (Becton Dickinson) antibodies and Mouse IgG2b-FITC model antibodies (Becton Dickinson) were used as a negative control to exclude non-specific staining (Fig. 3).

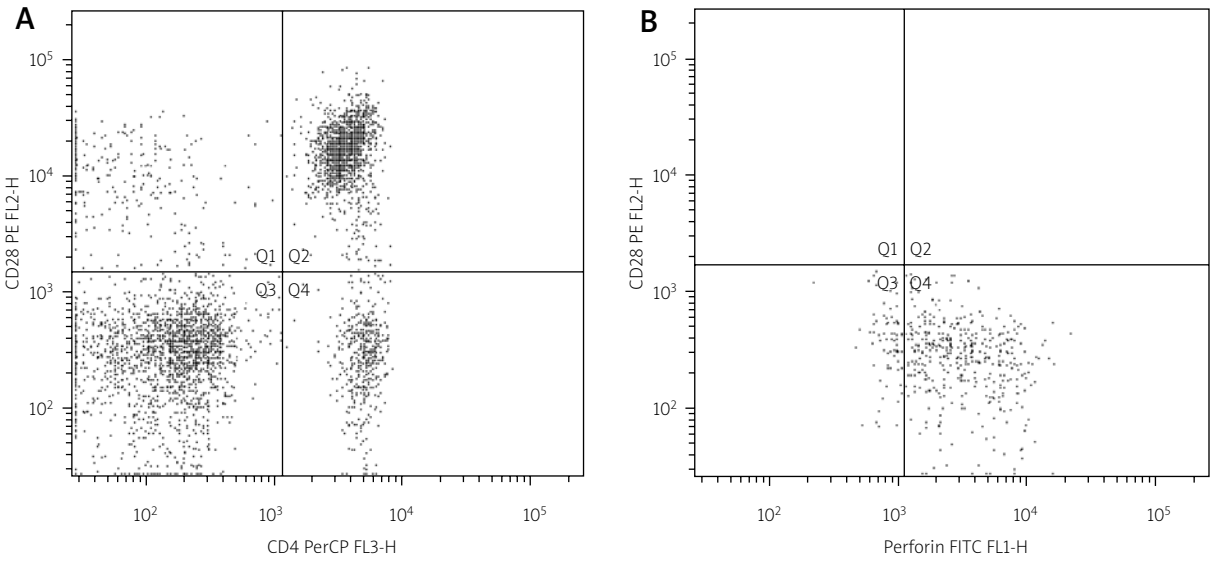

Fig. 2. Lymphocyte flow cytometry analysis with quadrant markers (tube no. 2). **A)** CD28 PE FL2-H – expression of CD28 PE-conjugated antibodies on analysed whole lymphocyte subpopulation. CD4 PerCP FL3-H – expression of CD4 PerCP-conjugated antibodies on analysed whole lymphocyte subpopulation. Q1 (upper left quadrant) – quadrant with $CD4^{-}CD28^{+}$ lymphocytes. Q2 (upper right quadrant) – quadrant with $CD4^{+}CD28^{+}$ lymphocytes. Q3 (lower left quadrant) – quadrant with $CD4^{-}CD28^{-}$ lymphocytes. Q4 (lower right quadrant) – quadrant with $CD4^{+}CD28^{-}$ lymphocytes. **B)** CD28 PE FL2-H – expression of CD28 PE-conjugated antibodies on differentiated $CD4^{+}$ lymphocyte subpopulation. Perforin FITC FL1-H – expression of Anti-Human Perforin FITC-conjugated antibodies on differentiated $CD4^{+}$ lymphocyte subpopulation. Q1 (upper left quadrant) – quadrant with $CD4^{+}CD28^{+}Perforin^{-}$ lymphocytes. Q2 (upper right quadrant) – quadrant with $CD4^{+}CD28^{+}Perforin^{+}$ lymphocytes. Q3 (lower left quadrant) – quadrant with $CD4^{+}CD28^{-}Perforin^{-}$ lymphocytes. Q4 (lower right quadrant) – quadrant with $CD4^{+}CD28^{-}Perforin^{+}$ lymphocytes.

The study was conducted upon the approval issued by the Bioethical Commission (BN resolution no. 001/36/06). Informed consent has been obtained from each subject.

Statistical analysis

At the analysis of measurable variables, the following ones were presented: median (Me), minimum value (Min), maximum value (Max) and standard deviation (SD). The measurable variables showed distributions significantly departing from normal distribution (Shapiro-Wilk test, $p < 0.05$), which is why non-parametric tests were used. To show the significance of differences among more than two groups, ANOVA Kruskal-Wallis was used, and to compare two groups of patients, the Mann-Whitney *U* test. Nominal variables were compared with the use of the χ^2 test or its modifications with Yates's correction or the precise two-sided Fisher's test (for tables 2 × 2). For a one-factor and then multi-factor analysis of an odds ratio (OR) with 95% confidence interval (95% CI), logistic regression was applied. As a statistical significance threshold, $p < 0.05$ was assumed. Statistical calculations were made with the use of the Statistica 7.1 programme.

Results

Demographical characteristics of the studied patients, with reference to the symptoms of the operated lesions, are set out in Table I.

Percentage of $CD4^{+}CD28^{-}$ lymphocytes in peripheral blood and their cytotoxicity vs. symptomatic nature of the plaques (plaque ipsilateral to the stroke)

No significant difference was found between the symptomatic plaque group and the asymptomatic plaque one when a global proportion of $CD4^{+}CD28^{-}$ Lc and a proportion of cytotoxic lymphocytes were considered (Table II).

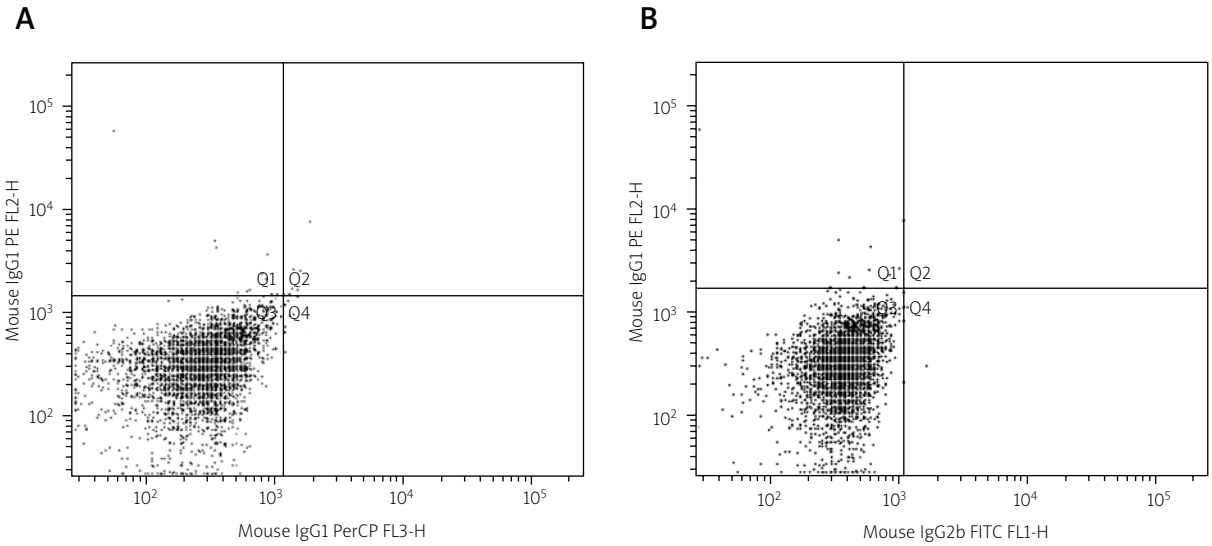

Fig. 3. Lymphocyte flow cytometry analysis with quadrant markers (tube no. 1). **A)** Mouse IgG1 PE FL2-H – expression of Mouse IgG1 PE-conjugated antibodies on analyzed whole lymphocyte subpopulation. Mouse IgG1 PerCP FL3-H – expression of Mouse IG1 PerCP-conjugated antibodies on analyzed whole lymphocyte subpopulation. Q1 (upper left quadrant) – lymphocytes positive for Mouse IgG1 PE, negative for Mouse IgG1 PerCP antibodies. Q2 (upper right quadrant) – lymphocytes positive for both types of control mouse antibodies. Q3 (lower left quadrant) – lymphocytes negative for both types of control mouse antibodies. Q4 (lower right quadrant) – lymphocytes negative for Mouse IgG1 PE antibodies, positive for Mouse IgG1 PerCP ones. **B)** Mouse IgG1 PE FL2-H – expression of Mouse IgG1 PE-conjugated antibodies on differentiated $CD4^+$ lymphocyte subpopulation. Mouse IgG2b FITC FL1-H – expression of Mouse IgG2b FITC-conjugated antibodies on differentiated $CD4^+$ lymphocyte subpopulation. Q1 (upper left quadrant) – lymphocytes positive for Mouse IgG1 PE, negative for Mouse IgG2b FITC antibodies. Q2 (upper right quadrant) – lymphocytes positive for both types of control mouse antibodies. Q3 (lower left quadrant) – lymphocytes negative for both types of control mouse antibodies. Q4 (lower right quadrant) – lymphocytes negative for Mouse IgG1 PE antibodies, positive for Mouse IgG2b FITC ones.

Table I. Demographical data of patients with symptomatic and asymptomatic atherosclerotic plaques

Atherosclerotic plaques of carotid arteries	Symptomatic	Asymptomatic	*p*
Number of patients (*n*)	46	45	0.9319
Age of patients (in years)	67.9	67.7	0.9114
Women, *n* (%)	14 (27.4)	11 (28.9)	0.6395
Men, *n* (%)	32 (72.6)	34 (71.1)	0.7984
Comorbidities (*n*)			
HA	46	26	0.0881
DM t. 2	15	10	0.4040
IHD	11	23	0.0702
PAD	12	11	0.8898
Dyslipidaemia	11	5	0.1805
Degree of stenosis (median)	80%	80%	1.000

HA – arterial hypertension, DM t. 2 – type 2 diabetes, IHD – ischaemic heart disease, PAD – obliterative atherosclerosis of the lower extremities

Table II. CD4+CD28– Lc and their cytotoxicity vs. symptomatic nature of carotid plaques

Atherosclerotic plaques of carotid arteries	Symptomatic	Asymptomatic	*p*
CD4+CD28– Lc (%)	3.55 (0.08-50.5)	6.13 (0.37-27.43)	0.1596
Perforin+CD4+CD28– Lc (%)	79.825 (0-99.75)	72.72 (1.73-100)	0.4777

CD4+CD28– Lc (%) – percentage of CD4+CD28– lymphocytes in peripheral blood, Perforin+CD4+CD28– Lc (%) – percentage of Perforin+CD4+CD28– lymphocytes in peripheral blood, Symptomatic – patients with symptomatic carotid plaques, Asymptomatic – patients with asymptomatic carotid plaques, Degree of stenosis – degree of carotid artery stenosis

Percentage of CD4+CD28– lymphocytes in peripheral blood and their cytotoxicity vs. instability of atherosclerotic plaques

In the course of a microscopic examination, 54 of the evaluated atherosclerotic lesions met the instability criteria set. In 18 cases, plaques were qualified as potentially unstable. The nature of the remaining 19 plaques was stable (Fig. 4). No significant relation between the proportion of CD4+CD28– Lc and their cytotoxicity and the nature of the studied plaques was observed (Table III).

Percentage of CD4+CD28– lymphocytes in peripheral blood and their cytotoxicity vs. individual histopathological parameters of the plaques (Tables IV and V)

a) CD4+CD28– Lc vs. mural thrombus

The proportion of CD4+CD28– Lc did not constitute a feature that would distinguish patients with plaques complicated by a mural thrombus or lack thereof.

The cytotoxicity of the cells in the former group was higher; however, the difference was not significant.

b) CD4+CD28– Lc vs. intraplaque haemorrhage

Intraplaque haemorrhages were observed in 15 cases. The percentage of CD4+CD28– lymphocytes in peripheral blood of such patients as well as the cytotoxicity of the lymphocytes were comparable to those observed in patients without haemorrhages.

c) CD4+CD28– Lc vs. inflammatory infiltrations

No significant difference in the proportion of the lymphocytes and their cytotoxicity depending on the size of inflammatory infiltrations in the investigated plaques was found.

d) CD4+CD28– Lc vs. plaque vessels

Patients with numerous vessels within atherosclerotic plaques were characterised by a higher percentage of CD4+CD28– Lc when compared to other patients (6.06% vs. 2.57%); nevertheless, the difference was not pronounced. The percentage of cytotoxic lymphocytes was also comparable.

e) CD4+CD28– Lc vs. foam cells

The percentage of lymphocytes, including the percentage of cytotoxic cells, was comparable in patients with both a high and low share of foam cells in the plaque structure.

f) CD4+CD28– Lc vs. plaque fibrous component

In the course of plaque fibrous component analysis, a significantly higher global percentage of the lymphocytes under research was found both in the group of patients with plaques containing mainly collagen fibres and in patients with plaques that were composed of equal proportions of collagen and elastin fibres.

Lymphocyte cytotoxicity was found to have no effect.

g) CD4+CD28– Lc vs. presence of cholesterol crystals

The percentage of the studied cells in peripheral blood was comparable in patients with and without cholesterol crystals present in the plaques. On the other hand, the lymphocyte cytotoxicity was significantly higher in patients with cholesterol crystals.

h) CD4+CD28– Lc vs. intraplaque calcification

No relation between the percentage of lymphocytes in the blood and the degree of calcification of the analysed plaques was observed. However, the percentage of cytotoxic lymphocytes was significantly higher in patients with lower plaque calcification.

A multifactor regression analysis of a dependent variable presented a strong relation between the cytotoxicity of CD4+CD28– lymphocytes and the presence of cholesterol crystals in the plaque ($p = 0.0178$). No relationship was established in the case of the remaining histopathological plaque parameters.

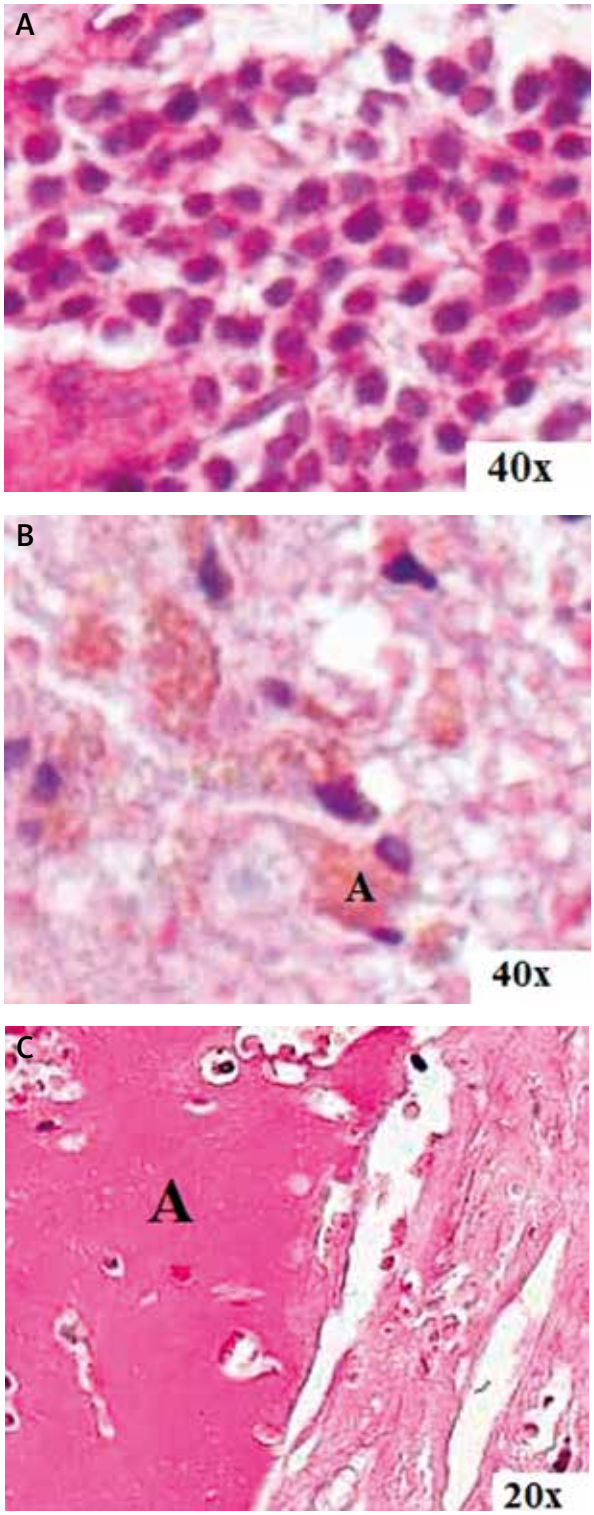

Fig. 4. Stable atherosclerotic plaques. **A)** massive inflammatory infiltrations, **B)** hemosiderin-laden macrophages – A, **C)** mass of thrombus – A

Discussion

The percentage of $CD4^+CD28^-$ lymphocytes is increased in the blood of patients with an acute atherosclerosis-related ischemic stroke [19]. Earlier studies stated that their presence in the blood is not a consequence of a stroke. Their high percentage is comparable to that observed in the no-stroke patients with atherogenic risk factors. In both groups, the proportion of the studied lymphocytes in the blood is significantly higher than that of a healthy population [19]. Current results yield similar conclusions. Therefore, the role of $CD4^+CD28^-$ Lc in ischemic stroke pathogenesis has not been ruled out. They still need to be treated as a factor promoting the development of an acute focal ischemia in the brain.

The presented material included patients with an advanced carotid arteries atheromatosis.

The percentage of $CD4^+CD28^-$ Lc in the blood was equally high regardless of the type of the analysed risk factors for stroke [20]. These results validate our previous observations and justify the search for potential relations between lymphocytes and atherosclerosis of extracranial arteries [6].

$CD4^+CD28^-$ Lc, especially their cytotoxic forms, promote atherosclerotic plaque destabilization [12,15]. It seems that these cells in themselves may lead to the stroke (i.e. to symptomatic plaques) [5,22]. In our study. the percentage of $CD4^+CD28^-$ Lc and their cytotoxicity were comparable in patients with symptomatic and asymptomatic plaques. Therefore, analysed cells are not prognostic factors for stroke (i.e. for symptomatic lesions within carotid arteries).

Research, including our previous studies, also shows that the symptomatic nature of carotid atherosclerotic plaques is rather an outcome of a combined effect of numerous, both intra- and extravascular factors [10,14]. $CD4^+CD28^-$ Lc could have been only one of such factors by their involvement in the process of plaque destabilization.

A relation of aforesaid circulated lymphocytes to less plaques' calcification may be an argument for that [17,24,28].

The results indicate a strong relation between the proportion of cytotoxic $CD4^+CD28^-$ Lc in the blood and the presence of cholesterol crystals in the plaques. Cholesterol crystals within atherosclerotic lesions are recognized as a potent factor promoting inflammation in the wall of an arterial vessel, which results in a cascade of events leading to plaque destabilization

Table III. CD4+CD28– Lc and their cytotoxicity vs. instability of atherosclerotic plaques in a microscopic analysis

Atherosclerotic plaques of carotid arteries	Unstable	Potentially unstable	Stable	*p*
CD4+CD28– Lc (%)	4.65 (0.14-50.5)	6.0 (0.2-33.24)	3.66 (0.08-28.91)	0.5503
Perforin+CD4+CD28– Lc (%)	80 (1.73-100)	61.97 (2.97-96.82)	82.2 (0-98.34)	0.3939

Unstable – patients with unstable carotid plaques, Potentially unstable – patients with potentially unstable carotid plaque, Stable – patients with stable carotid plaques, CD4+CD28– Lc (%) – percentage of CD4+CD28– lymphocytes in peripheral blood, Perforin+CD4+CD28– Lc(%) – percentage of Perforin+CD4+CD28– lymphocytes in peripheral blood

Table IV. Percentage of CD4+CD28– Lc in peripheral blood vs. microscopic evaluation of carotid atherosclerotic plaques

Histopathological parameter	*n*	CD4+CD28– Lc (%)	*p*
Cholesterol crystals			
Yes	53	4.19 (0.14-50.5)	0.3762
No	38	6.425 (0.08-28.91)	
Foam cells			
Numerous	59	5.02 (0.08-50.5)	0.3009
Scarce	32	3.97 (0.14-33.24)	
Mural thrombus			
Yes	54	4.65 (0.14-50.5)	0.2873
No	37	3.83 (0.08-33.24)	
Calcifications			
Numerous	40	3.77 (0.08-50.5)	0.5340
A few minor ones	26	5.76 (0.34-28.91)	
Isolated minor ones	25	6.12 (0.24-29.09)	
Vessels			
Numerous	66	6.06 (0-100)	0.2822
Isolated large focus	17	1.78 (0-100)	
Isolated minor focus	8	3.21 (0.24-17.84)	
Fibrous component			
Majority of elastin fibres	42	2.755 (0-100)	0.0119
Majority of collagen fibres	2	17.225 (0-100)	
Equal proportion of collagen and elastin fibres	47	6.14 (0.08-100)	
Inflammatory infiltrations			
Massive	16	6.71 (0.24-27.43)	0.7560
Widespread	56	4.11 (0.14-50.5)	
Isolated minor ones	19	4.52 (0.08-29.09)	
Intraplaque haemorrhages			
Yes	16	6.13 (0.24-100)	0.4606
No	75	4.19 (0-100)	

Histopathological parameter – evaluated histopathological parameter of carotid atherosclerotic plaque, n – number of patients with appropriate plaque feature, CD4+CD28– Lc (%) – percentage of CD4+CD28- lymphocytes in peripheral blood, Cholesterol crystals – presence of cholesterol crystals in atherosclerotic plaque, Foam cells – foam cells in atherosclerotic plaque, Mural thrombus – presence of a mural thrombus in atherosclerotic plaque, Calcifications – calcifications in atherosclerotic plaque, Vessels – vessels in atherosclerotic plaque, Fibrous component – fibrous element structure in atherosclerotic plaque, Inflammatory infiltrations – inflammatory infiltrations in atherosclerotic plaque, Intraplaque haemorrhages – intraplaque haemorrhages present in atherosclerotic plaque

Table V. Cytotoxicity of $CD4^+CD28^-$ Lc in peripheral blood vs. microscopic evaluation of carotid atherosclerotic plaques

Histopathological parameters of the plaques	*n*	$Perforin^+CD4^+CD28^-$ Lc (%)	*p*
Cholesterol crystals			
Yes	53	81.905 (2.22-100)	0.0439
No	38	66.67 (0-98.6)	
Foam cells			
Numerous	59	79.13 (0-100)	0.6249
Scarce	32	74.85 (2.22-100)	
Mural thrombus			
Yes	54	80 (1.73-100)	0.3928
No	37	71.875 (0-98.34)	
Calcifications			
Numerous	40	69.23 (2.62-99.75)	0.0541
A few minor ones	26	72.72 (1.73-97.33)	
Isolated minor ones	25	88.09 (0-100)	
Vessels			
Numerous	66	68.485 (0-100)	0.5665
Isolated large focus	17	73.53 (0-99.75)	
Isolated minor focus	8	75.425 (0-98.34)	
Fibrous component			
Majority of elastin fibres	42	50 (0-100)	0.1971
Majority of collagen fibres	2	42.41 (2.62-82.2)	
Equal proportion of collagen and elastin fibres	47	80.95 (0-100)	
Inflammatory infiltrations			
Massive	16	75.49 (2.22-100)	0.2874
Widespread	56	74.19 (0-100)	
Isolated minor ones	19	86.95 (25.58-98.34)	
Intraplaque haemorrhages			
Yes	16	68.305 (0-96.3)	0.6126
No	75	68.99 (0-100)	

Histopathological parameter – evaluated histopathological parameter of carotid atherosclerotic plaque, n – number of patients with appropriate plaque feature, $Perforin^+CD4^+CD28^-$ (%) – percentage of $Perforin^+CD4^+CD28^-$ lymphocytes in peripheral blood, Cholesterol crystals – presence of cholesterol crystals in atherosclerotic plaque, Foam cells – foam cells in atherosclerotic plaque, Mural thrombus – presence of a mural thrombus in atherosclerotic plaque, Calcifications – calcifications in atherosclerotic plaque, Vessels – vessels in atherosclerotic plaque, Fibrous component – fibrous element structure in atherosclerotic plaque, Inflammatory infiltrations – inflammatory infiltrations in atherosclerotic plaque, Intraplaque haemorrhages – intraplaque haemorrhages present in atherosclerotic plaque

[1,4,25]. Other researchers suggest that $CD4^+CD28^-$ Lc appear originally in the blood and then infiltrate tissues, including atheromatous lesions, in which they enhance inflammatory mechanisms [27].

Therefore, one may suspect that cytotoxic forms of $CD4^+CD28^-$ Lc indirectly favour plaque destabilization by inflammatory process enhancement, maybe as a reaction to the intraplaque pro-inflammatory cholesterol deposits. However, this thesis needs additional research.

An identification of these cells directly inside carotid plaques may provide more information on

the participation of CD4+CD28– Lc in the stroke pathogenesis.

Conclusions

CD4+CD28– cytotoxic lymphocytes seem to be involved in the development of carotid atherosclerotic plaques.

Intraplaque cholesterol deposits may contribute to this process.

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