Abstract

Background and purpose: CD4+CD28– lymphocytes can directly contribute to the instability of atherosclerotic plaque. This paper attempts to answer the question of the potential influence of the CD4+CD28– lymphocyte population on the ultrasound image of atherosclerotic plaque in the common carotid artery (CCA) wall.

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 mentioned risk factors, without ischaemic stroke. The control group comprised 33 healthy individuals. The percentage of CD4+CD28– lymphocytes was assessed with flow cytometry.

Results: A significant difference in the incidence of heterogeneous plaques was noted between groups 1 and 3 (p = 0.0023) as well as between group 2 and 3 (p = 0.0005), whereas groups 1 and 2 did not differ from each other. The proportion of CD4+CD28– lymphocytes was similar in groups 1 and 2 (p = 0.97), but it differed between groups 1 and 3 (p < 0.0001) and between groups 2 and 3 (p < 0.001).

Streszczenie


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 (grupo 1.). Grupę 2. stanowiło 34 chorych z wymienionymi czynnikami ryzyka, bez udaru, a grupę kontrolną – 33 osoby uznane za zdrowe. Odsetek limfocytów CD4+CD28– we krwi obwodowej analizowano za pomocą cytometrii przepływowej.

 Wyniki: Wykazano istotną różnicę w częstości występowania blaszek heterogennych pomiędzy grupami 1 i 3 (p = 0.0023) oraz 2 i 3. (p = 0.0005), natomiast grupy 1 i 2. nie różniły się istotnie pod względem odsetka osób z tego rodzaju blaszkami. Odsetek badanych limfocytów CD4+CD28– w grupach 1 i 2. był zbliżony (p = 0.97), natomiast istotnie większy w porównaniu do grupy 3.
A correlation was found between the proportion of CD4+CD28− lymphocytes in the blood and the number of CCA atherosclerotic plaques ($R_s = 0.191$, $p = 0.046$). The proportion of CD4+CD28− lymphocytes in peripheral blood did not correlate with the ultrasound types of atherosclerotic plaques. No correlation between the proportion of CD4+CD28− lymphocytes and the area of atherosclerotic plaques was found.

**Conclusions:** The correlation between the proportion of CD4+CD28− lymphocytes and the number of atherosclerotic plaques within the CCA suggests that the cells are involved in the mechanism of carotid plaque formation. There is no proof of the involvement of the above-mentioned cells in the mechanism of plaque destabilization in those arteries.

**Key words:** ischaemic stroke, atherosclerotic plaque, common carotid artery, ultrasonography, CD4+CD28− lymphocytes.

**Introduction**

About half of all ischaemic cerebral infarctions are caused by atherosclerotic alterations occurring in extracranial arteries, and less frequently so in large intracranial arteries. According to the currently adopted uniform theory of atherosclerosis, it results from complex interactions between the vascular wall cells, blood cells and plasma lipoproteins. Consequently, the endothelial function is disturbed, blood platelets become activated, and retention, oxidation and aggregation of lipoproteins occur in the vascular wall. Repeated damage to the endothelium initiates a chronic inflammatory-fibroproliferative response of the vascular wall [1-3].

It has been proven that in the early phase of atherosclerotic alterations CD4+ lymphocytes, which form part of the inflammatory infiltration in the vessel wall, become activated. Those cells enhance the inflammatory response in the vascular wall [4-7]. Special significance is attributed to the CD4+ lymphocyte subpopulation, which is devoid of the CD28 receptor (LcCD4+CD28−). They are subject to activation despite the lack of stimulation by B7/CD28 [5,6]. The cells can indirectly contribute to the instability of atherosclerotic plaque within coronary arteries by creating a microenvironment in the plaque which consists of a large number of pro-inflammatory cytokines, especially interferon γ, which is a potential stimulator of macrophages [8-11]. It appears that LcCD4+CD28− directly contribute to the destabilization and rupture of atherosclerotic plaque in different pathways. The correlation between the number of LcCD4+CD28− and the presence of atherosclerotic alterations in the ultrasound examination was analysed in patients with rheumatoid arthritis, a condition which itself can be conducive to the growth of the subpopulation of those lymphocytes, including in the atherosclerotic plaque [12]. The involvement of the LcCD4+CD28− subpopulation in the pathogenesis of ischaemic stroke has so far been discussed in very few papers [13].

Determination of the temporal relation between the onset of a stroke episode and the accompanying growth of the subpopulation of those cells in the blood fails to resolve a series of problems. One of the most important ones is the answer to the following question: does the increase in the proportion of LcCD4+CD28− in the blood at the initial stage of acute ischaemic stroke stem from an immunological-inflammatory response to nervous tissue necrosis, or does it point to the involvement of those cells in the occurrence of carotid plaques? This prompted us to undertake studies on the mentioned problems, with reference to the plaque ultrasound appearance.

**Material and methods**

One-hundred and nine persons (56 males and 53 females), aged 45 to 65 years, were qualified for the study and divided into three groups.

Inclusion and exclusion criteria, criteria pertaining to the diagnosis of the analysed ischaemic stroke risk factors and details regarding other examinations, including the method of determining the expression of the CD28 receptor on the surface of LcCD4+, have been presented in part I of the paper [14].
Doppler ultrasound examination of extracranial arteries was performed at the ultrasound laboratory of the Neurological Department using the ESAOTE AU 5 device equipped with a 7.5 MHz linear probe. Thickness and echogenicity of the common carotid artery (CCA) was analysed with the assumption that those parameters reflect the accumulated influence of all the atherosclerosis risk factors. The examination covered both CCAs. The artery was divided into 2 segments in accordance with the Mannheim Intima-Media Thickness Consensus [15]. 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, anterolateral and posterolateral and sagittal, so as to evaluate the aspect of the vessel and its patency. The CCA posterior wall intima-media thickness (IMT) was measured in B-mode projection. The location, number and size of atherosclerotic plaques were registered.

Atherosclerotic plaques were defined in accordance with the guidelines of the Mannheim Intima-Media Thickness Consensus. Atherosclerotic plaque is a focal structure that encroaches into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface [15,16]. Atherosclerotic plaques were subjectively graded by two methods. The first was according to the relative contribution of echolucent and echogenic plaques using the classification by Gray-Weale et al. [17]. The second method classified the plaques as either homogeneous or heterogeneous [18]. The area of the cross-section of atherosclerotic plaques was determined by means of the method described by Spence et al. [19-21]. If more than one plaque was visualized in the same artery, the calculations were aggregated. Systolic and diastolic velocity of blood flow was also evaluated using the pulsed wave Doppler method so as not to overlook the presence of hypoechogenic atherosclerotic plaques in the examined region. Ultrasound imaging and IMT measurements were assessed by two persons. Intra-observer and inter-observer coefficients of variation were used, according to the Bland-Altman method [22]. Concordance for intra-observer and inter-observer variability for p-value was p < 0.0001. The data acquired were archived using the ‘SARO’ ultrasound image archiving system.

**Statistical analysis**

Measurable variables demonstrated distributions significantly different from the normal distribution (Shapiro-Wilk test, p < 0.05), which is why non-parametric tests were employed. In order to demonstrate the significance of the differences among more than two groups, Kruskal-Wallis ANOVA was used, and 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), the Wilcoxon pair sequence test was used. Correlation strength between the variables was evaluated by means of the value of the Spearman rank correlation coefficient (R). Nominal variables were compared using the χ² test or Fisher two-tailed 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**

Atherosclerotic plaques were reported in 20 people (47.6%) from group 1, in 19 people (55.9%) from group 2, and in 6 people from the control group (19.8%).

<table>
<thead>
<tr>
<th>Type of atherosclerotic plaques</th>
<th>Patients with ischaemic stroke (Group 1) (n = 42)</th>
<th>Patients with arterial hypertension and/or diabetes mellitus type 2 (Group 2) (n = 34)</th>
<th>Controls without arterial hypertension or diabetes mellitus type 2 (Group 3) (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of hyperechogenic plaques</td>
<td>15</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Number of heterogeneous plaques</td>
<td>28</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>Number of hypoechogenic plaques</td>
<td>–</td>
<td>3</td>
<td>–</td>
</tr>
</tbody>
</table>
The number of consecutive types of atherosclerotic plaques is provided in Table 1. The number of people with atherosclerotic plaques was similar in groups 1 and 2 \((p = 0.498; \text{Fisher exact test})\), while it differed between group 1 and group 3 \((p = 0.003)\) and between group 2 and group 3 \((p = 0.003)\). In groups 1 and 2, atherosclerotic plaques occurred significantly more often in CCA bifurcations than in the CCA \((p = 0.0007\) and \(p = 0.0006, \text{respectively}\)), while in group 3 no such difference was found \((p = 0.103; \text{Fisher exact test})\). A significant difference in the incidence of heterogeneous plaques was noted between groups 1 and 3 \((p = 0.0023)\) as well as between groups 2 and 3 \((p = 0.0005)\), whereas groups 1 and 2 were similar in this regard \((p = 0.4875; \chi^2\text{test})\). No hypoechogenic plaques were reported for patients from groups 1 and 2, and only for two patients \((5.88\%)\) from group 2 \((p = 0.106; \chi^2\text{test})\).

Mean area of atherosclerotic plaques in CCA of patients with stroke was 18 mm\(^2\) \((\text{range}: 11.8-172 \text{ mm}\(^2\))\), in group 2 it was 24 mm\(^2\) \((\text{range}: 11.7-116 \text{ mm}\(^2\))\), and in group 3 it was 3 mm\(^2\) \((12.8-26 \text{ mm}\(^2\))\). A significant difference in the area of atherosclerotic plaques was reported between groups 1 and 3 \((p = 0.001)\) as well as between groups 2 and 3 \((p = 0.0001)\), while there was no significant difference between groups 1 and 2 \((p = 0.27, \text{Mann-Whitney } U\text{-test})\).

Figure 1 presents the proportion of \(\text{LcCD4}^+\text{CD28}^-\) in the blood of the examined groups. There was no significant difference between the proportion of those lymphocytes in groups 1 and 2 \((p = 0.97)\). On the other hand, in both of the groups it was significantly higher than in group 3 \((\text{group 1 vs. group 3}: p < 0.0001; \text{group 2 vs. group 3}: p < 0.001)\).

The proportion of \(\text{LcCD4}^+\text{CD28}^-\) in the blood correlated with the number of CCA atherosclerotic plaques with no consideration for examined groups \((Rs = 0.191, p = 0.046)\). On the other hand, the proportion of \(\text{LcCD4}^+\text{CD28}^-\) in peripheral blood did not correlate with the ultrasound types of atherosclerotic plaques. No correlation between the proportion of \(\text{LcCD4}^+\text{CD28}^-\) lymphocytes and the area of atherosclerotic plaques was found (Table 2).

**Discussion**

The main structural indicators of atherosclerotic alterations are atherosclerotic plaques. We were primarily interested in the ultrasound features of the atherosclerotic plaques pointing to their instability (high-risk plaques). It is assumed that in carotid arteries unstable pla-

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**Table 2.** Correlations between the proportion of CD4\(^+\)CD28\(^-\) lymphocytes in peripheral blood and the ultrasound parameters of atherosclerotic plaques

<table>
<thead>
<tr>
<th>Correlation between proportion of CD4(^+)CD28(^-) and:</th>
<th>Patients with ischaemic stroke (Group 1) ((n = 42))</th>
<th>Patients with arterial hypertension and/or diabetes mellitus type 2 (Group 2) ((n = 34))</th>
<th>Controls without arterial hypertension or diabetes mellitus type 2 (Group 3) ((n = 33))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plaques</td>
<td>0.053 0.740</td>
<td>-0.007 0.964</td>
<td>-0.013 0.941</td>
</tr>
<tr>
<td>Left and right carotid plaques area</td>
<td>0.032 0.743</td>
<td>0.046 0.795</td>
<td>-0.028 0.877</td>
</tr>
<tr>
<td>Number of hyperechogenic plaques</td>
<td>0.046 0.772</td>
<td>0.004 0.981</td>
<td>-0.024 0.893</td>
</tr>
<tr>
<td>Number of heterogeneous plaques</td>
<td>-0.011 0.944</td>
<td>0.059 0.739</td>
<td>0.093 0.605</td>
</tr>
<tr>
<td>Number of hypoechogenic plaques</td>
<td>- -</td>
<td>-0.057 0.747</td>
<td>- -</td>
</tr>
</tbody>
</table>

\(Rs\) – Spearman rank correlation coefficient
ques, or plaques manifesting an increased risk of rupture, do not contain a large number of lipids and are more fibrotic [23]. Plaque rupture is caused by an intramural hematoma or dissection. They are heterogeneous in ultrasound images [24].

In our material, heterogeneous plaques predominated in both group 1 and group 2. This suggests that the main risk factors for ischaemic stroke – hypertension, type 2 diabetes, dyslipidaemia, as well as overweight – may contribute to unstable (heterogeneous) plaque formation in CCA. More and more significance is also attached to the inflammatory and immunological mechanisms involved in the pathogenesis of atherosclerosis and nervous tissue ischaemia.

Atherosclerotic plaques in the internal carotid artery of a patient with stroke symptoms demonstrate higher expression of inflammatory factors than the ones in the arteries of patients showing no signs of cerebral infarction [25].

The most important cells of the immunological system taking part in the atherosclerosis-related damage are monocytes – macrophages and lymphocytes [26-29]. We were especially interested in the LcCD4+ CD28− subpopulation. Those cells can hardly be found in healthy individuals. In persons below 40 years of age they constitute from 0.1 to 2.5% of LcCD4+ in peripheral blood [30]. In the group of patients with their first-ever ischaemic stroke, the proportion of those cells was significantly higher than in the control group.

The role played by LcCD4+ CD28− in the pathogenesis of cerebral infarction is not yet known [12,31]. Their high proportion in patients included in group 2 suggests that the cells are involved in the development of atherosclerotic lesions in CCA walls rather than in the processes reactive to nervous tissue ischaemia.

The question whether the increase in the proportion of LcCD4+ CD28− may lead to the destabilization of atherosclerotic plaques in the CCA remains open. We observed a significantly higher proportion of LcCD4+ CD28− in the patients with stroke, arterial hypertension, diabetes and atherogenic dyslipidaemia, as well as in the patients burdened with the above risk factors but with no cerebral infarction episode. Patients with heterogeneous plaques, considered unstable, predominated in both groups. However, we observed a correlation between the general number of atherosclerotic plaques and the proportion of LcCD4+ CD28−, but we did not find a direct correlation between the elevated proportion of LcCD4+ CD28− and the presence of heterogeneous plaques in the CCA wall.

Conclusions

1. The correlation between the proportion of CD4+ CD28− lymphocytes and the number of atherosclerotic plaques within the CCA suggests that the cells are involved in the mechanism of carotid plaque formation.

2. There is no proof of the involvement of the above-mentioned cells in the mechanism of plaque destabilization in those arteries.

Acknowledgements

The authors would like to thank Professor Anna Wałęcka, from the Department of Imaging Diagnostics and Intervention Radiology, Pomeranian Medical University, and Professor Wenancjusz Domagała, from the Pathomorphology Department of the Medical Faculty of Pomeranian Medical University.

Disclosure

Authors report no conflict of interest.

References


