EREDETI KÖZLEMÉNY

ORIGINAL ARTICLE

Cerebellar antibodies in post-stroke sera of acute ischemic stroke patients

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Background and purpose – Although serum anti-neuronal antibodies are found in acute ischemic stroke (AIS) patients, it is not completely clear whether they are already present before the cerebrovascular event or emerge thereafter.
Methods – Sera of 21 consecutive first-ever AIS patients were collected within the first day of AIS (baseline), as well as 1 and 6 months after AIS. Well-characterized and novel anti-neuronal antibodies were investigated by cell-based assays, immunoblotting and indirect immunohistochemistry.
Results – None of the AIS sera collected at different time points showed well-characterized antibodies. In 7 patients, 1- and 6-month sera (but not baseline sera) showed IgG mostly reacting with soma and dendrites of cerebellar Purkinje cells. Antibody-positive patients did not differ in terms of clinical and etiological features.
Conclusion – Our results provide evidence for the antibody-triggering action of AIS. Although anti-cerebellar antibodies are not associated with the severity of stroke, they may potentially contribute to chronic post-stroke complications and disability.

Keywords: stroke, ischemic stroke, antibody, cerebellar, autoimmunity

Kisagyi antitestek akut ischaemiás stroke-on átesett betegek szérumában
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Háttér és cél – Bár akut ischaemiás stroke-os (AIS) betegeknél előfordulnak a szérumban antineuronalis antitestek, nem teljesen világos, hogy ezek már a cerebrovascularis esemény előtt jelen vannak, vagy csak később alakulnak ki.
Módszerek – Huszonegy, első AIS-ében szenvedő beteg szérumát gyűjtöttük össze az AIS utáni első napon belül (kiindulási állapot), valamint 1 és 6 hónappal az AIS után. Jól jellemzett és új antineuronalis antitesteket vizsgáltunk sejtalapú vizsgálatokkal, immunoblottinggal és indirekt immunhisztokémiával.
Eredmények – A különböző időpontokban gyűjtött AIS-szérumok egyike sem mutatott jól jellemzett antitesteket. Hét betegnél az 1 és 6 hónapos szérumok (de nem a kiindulási szérumok) IgG-t mutattak, ami főként a kisagyi Purkinje-sejtek szómájával és dendritjeivel reagált. Az antitestpozitív betegek nem különböztek a klinikai és etiológiai jellemzők tekintetében.
Következtetés – Eredményeink bizonyítékot szolgáltatnak az AIS antitest-indukáló hatására. Bár a kisagyellenes antitestek nem állnak összefüggésben a stroke súlyosságával, potenciálisan hozzájárulhatnak a stroke utáni krónikus szövődményekhez és a rokkantsághoz.

Kulcsszavak: stroke, ischaemiás stroke, antitest, kisagyi, autoimmunitás

Acute ischemic stroke (AIS) causes damage to neurons and glia causing disruption of the blood-brain barrier and release of damage associated proteins. These events facilitate activation of the resident innate immune cells of the brain, infiltration of immune cells into the central nervous system and access of brain-derived antigens into the lymphoid tissue[1]. Overall, these factors result in activation of the adaptive immune system characterized by activated T and B cells, increased production of IgG, IgM and IgA in cerebrospinal fluid and emergence of antibody-producing B cells in the brain[1, 2].

Adaptive immune system activation may also lead to production of anti-neuronal antibodies, which have been reported to be associated with impaired cognition, increased clinical severity of stroke and infarct size[3, 4]. However, it is still not entirely clear whether these immunoglobulins merely represent pre-existing and naturally occurring antibodies or AIS may actually trigger the production of novel anti-neuronal IgG.

Materials and methods

Participants

We consecutively enrolled 21 first-ever AIS patients admitted to our inpatient clinic within a few hours after the onset of cerebrovascular event. Clinical scores, clinical severity scores and inflammation-related blood count/biochemistry parameters (white blood cells [WBC], lymphocytes, neutrophils, C-reactive protein [CRP], sedimentation) were noted **(Table 1)**. AIS patients with a previous history of stroke or any other neurological disorder, with any coexisting disease or infection (shortly before AIS and during the 6-month follow-up period) and under immunosuppressive treatment (before AIS or during the 6-month follow-up period) were excluded. Also, patients showing serum anti-neuronal antibodies in baseline sera were not included. Sera of 30 age/gender matched healthy volunteers were used as controls in antibody assays.

AIS was diagnosed on the basis of clinical features and cranial MRI (T1-, T2-, FLAIR- and diffusion-weighted) findings. All AIS patients underwent Doppler ultrasonography of the carotid arteries, electrocardiogram (ECG), transthoracic echocardiogram, 24-hour Holter monitoring, cranial and cervical computed tomography angiography (CTA) investigations on a routine basis. Patients with no pathological findings in these investigations were considered as AIS of undetermined etiology. Intracranial hemorrhage was ruled out by neuroimaging in all participants. All patients received a standard treatment protocol, as per international guidelines for AIS management[5]. Ethical approval was obtained from the Institutional Review Board.

Antibody assays

Sera of AIS patients were collected within the first day of stroke (baseline sample), 1 month and 6 months after AIS and were kept frozen at -80°C until use. Well-characterized anti-neuronal antibodies were investigated in 1:30 diluted sera by commercial kits (Euroimmun, Luebeck, Germany) using cell-based (NMDA receptor, LGI1, CASPR2, $GABA_B$ receptor, AMPA receptor, glutamic acid decarboxylase antibodies) or immunoblotting (Yo and Tr/DNER antibodies) assays. To investigate anti-neuronal antibodies directed against novel antigens, indirect immunohistochemistry was performed with frozen 10-µm-thick sections of rat brain fixed in paraformaldehyde overnight, using patient and control sera (1:200, overnight incubation at 4°C), secondary biotinylated anti-human IgG (1:1000, 2 h at room temperature) and the avidin-biotin-peroxidase method[6]. Brain sections incubated with healthy human sera and only secondary anti-IgG were used as controls. Intensity of immunolabeling was scored visually on a range from 0 (negative) to 4 (very strong) by two independent observers. In cases the scores of the two observers did not overlap, we took the average of two separate scores.

Statistics

Data of anti-neuronal antibody positive and negative patients were compared by Student's t, Mann Whitney U or chi-square tests, as required. $p < 0.05$ was considered statistically significant.

Results

Cerebellar antibodies in AIS

None of the AIS patients showed well-characterized antibodies in sera collected at baseline, 1 and 6 months after the cerebrovascular accident. Likewise, baseline serum samples of AIS patients did not show any specific staining pattern on frozen rat brain sections. By contrast, both 1st and 6th month serum IgGs of 7 AIS patients reacted with the dendritic projections and soma of Purkinje cells and the molecular and granular layers of the cerebellum **(Figure 1)**. Serum IgG of AIS patients did not react with the white matter of the cerebellum. Intensity of immunolabeling was scored through assessment of 5 randomly selected cerebellar cortex fields under the microscope for each participant. There was no difference between 1st (mean, 3.6±0.5; range, 3-4; median, 4) and 6th month (3.4±0.5; 3-4; 3) serum samples of 7 antibody positive AIS patients in terms of intensity and binding pattern (p=0.313 by Mann-Whitney U). By contrast, antibody positive AIS patients showed significantly higher IgG

Table 1. *Comparing characteristics of acute ischemic stroke patients with and without cerebellar neuropil antibodies (Ab)*

	Neuropil-Ab positive (n=7)	Neuropil-Ab negative (n=14)	p value
Age	66.1 ± 11.8	68.6 ± 11.3	0.325*
Sex (men/women)	5/2	9/5	0.743**
TOAST (n)			
Large artery atherosclerosis	1	2	0.732**
Cardioembolism	3	6	
Small-vessel occlusion	0	2	
Undetermined etiology	3	4	
Other determined etiology	0	0	
Large vessel occlusion	0	0	
OCPS classification (n)			
TACI	4	4	0.392**
PACI	3	6	
POCI	0	2	
LACI	0	2	
Localization (n)			
MCA	7	11	0.417**
PCA	0	2	
BA	0	1	
Brainstem infarct (n, %)	0 (0%)	2 (14%)	0.293**
Cerebellar infarct (n, %)	0 (0%)	0 (0%)	NA**
Vascular risk factors (n)			
Hypertension	4	12	0.945**
Type 2 diabetes mellitus	3	8	
Coronary artery disease	1	3	
Atrial fibrillation	1	1	
Hyperlipidemia	0	1	
Cigarette use	1	3	
Congestive heart failure	0	2	
ICU admission (n, %)	4 (57%)	7 (50%)	0.757**
Death in ICU (n, %)	0 (0%)	1 (7%)	0.469**
Maximum NIHSS	10.1 ± 8.1	8.9 ± 5.0	0.355***
Maximum mRS	3.7 ± 1.6	3.9 ± 1.4	0.422***
Increased inflammation findings (n, %)	5 (71%)	3 (21%)	0.026**

TOAST: trial of ORG 10172 in acute stroke treatment classification, ICU: intensive care unit, NIHSS: National Institutes of Health Stroke Scale, mRS: modified Rankin scale, OCPS: the Oxfordshire Community Stroke Project, LACI: lacunar infarct, PACI: partial anterior circulation infarct, TACI: total anterior circulation infarct, POCI: posterior circulation infarct, MCA: medical cerebral artery, PCA: posterior cerebral artery, BA: basilar artery, n: number, NA: not applicable.
Numeric data is presented as average ± standard deviation. Significant p value is denoted by bold characters.
* Student's t-test, ** chi-square test, *** Mann Whitney U test.

binding scores than the remaining AIS patients (0.4±0.1; 0-1; 0; p<0.001) and the healthy controls (0.3±0.1; 0-1; 0; p<0.001). No appreciable staining was observed in other parts of the rat brain. Serum IgG of healthy controls did not react with brain sections.

Comparison of anti-cerebellar antibody positive and negative patients

AIS patients with and without cerebellar antibodies did not differ in terms of stroke etiology, vascular risk factors, prevalence of ICU admission/death and clinical severity scores of stroke. All AIS patients with anti-cerebellar antibodies had partial or total anterior circulation infarcts due to the occlusion of the middle cerebral artery. Since they had moderate to severe hemiplegia, cerebellar signs and symptoms could not be assessed on the hemiplegic body side. No cerebellar signs were elicited on the non-hemiplegic sides of the antibody-positive patients during the initial admission or 6-month follow-up. Notably, one of the patients with posterior circulation-associated AIS (Table 1) exhibited a cerebellar lacunar infarct. However,

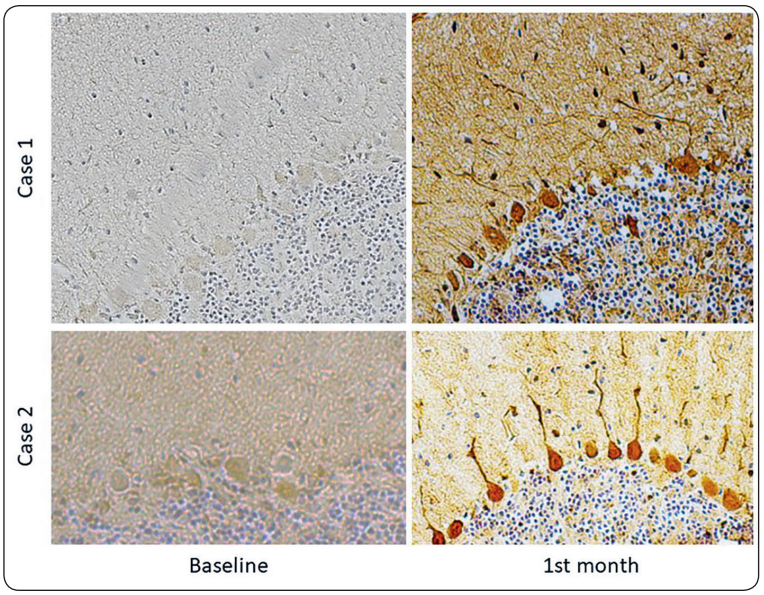

Figure 1. *Section of rat cerebellum immunolabeled with sera of two acute ischemic stroke patients. IgG of sera obtained at the 1st month of follow-up shows intense reactivity with the somata and dendritic projections of Purkinje cells and with the molecular and granular layers of the cerebellum (right panels), whereas those obtained from the same patients shortly after stroke occurrence (baseline) do not show an appreciable immunoreactivity with the brain tissue (left panels). Staining was performed with the avidin-biotin-peroxidase technique (brown) with hematoxylin counterstaining (blue); original magnification x20*

this patient exhibited neither cerebellar symptoms nor anti-cerebellar antibodies. Eight AIS patients showed mildly elevated sedimentation, CRP and WBC. Significantly higher ratio of AIS patients with cerebellar antibodies displayed increased sedimentation (1 antibody-positive), CRP (3 antibody-positive, 2 antibody-negative) or WBC (2 antibody-positive, 1 antibody-negative) levels than antibody negative patients **(Table 1)**. None of the AIS patients showed altered lymphocyte or neutrophil counts.

Discussion

Our results indicate that AIS is associated with the presence of circulating anti-neuronal antibodies, in line with previous studies[3, 4, 7–9]. However, these studies have often used the ELISA method which might detect antibodies to non-linear epitopes in both patient and healthy control groups. Also, previous studies have often found IgA/IgM antibodies to neuronal surface antigens, which are often not pathogenic and might be found in non-immune disorders[10]. Most importantly, these antibodies were mostly detected in the first few hours to days of the post-stroke period[8, 9]. Production of IgG antibodies usually takes several weeks. Also, in the rodent model of stroke, antibody producing B cells emerge 2 weeks following stroke occurrence[1]. Thus, stroke-associated antibodies reported so far putatively represent rapid amplification of pre-existing naturally occurring antibodies[1].

To investigate the antibody boosting effect of AIS, we collected baseline and post-stroke sera and included only patients who had not displayed antibodies shortly after AIS. Emergence of anti-neuronal IgGs with similar cerebellar staining pattern 1 month after the cerebrovascular event indicates that AIS promotes production of antibodies directed mostly against the layers of the cerebellar cortex. This effect is not temporary and persists at least for 6 months indicating the presence of long-lived plasma cells and memory cells. In a recent study with a similar design[9], authors have shown a very similar cerebellar staining pattern with the sera of AIS patients lending further support to our results. However, authors have failed to detect novel anti-neuronal antibodies in the post-stroke 30- and 90-day samples. This discrepancy might be due to the sensitivity difference between the immunofluorescence method used in the previous study and the indirect immunohistochemistry method used in the present study. Since minute details of the immunofluorescence method have not been provided, a thorough comparison is not possible. However, we believe that overnight fixation with paraformaldehyde and overnight incubation with patient sera might have increased the chances of detecting cell membrane antibodies and low-affinity antibodies, respectively.

A drawback of our study was unavailability of AIS patients with pure cerebellar infarcts. However, none of the seropositive patients of our cohort had a cerebellar or brainstem infarct, suggesting that antibodies did not necessarily occur as a result of increased exposure of the immune system to cerebellar antigens. Also, the single patient with a cerebellar lacunar infarct did not display cerebellar antibodies. It is fairly possible that anti-cerebellar antibodies found in our study develop against a different antigen and merely cross-react with antigen(s) found predominantly in the cerebellar tissue.

The immunostaining pattern obtained in our study is somewhat reminiscent of the so-called "Medusa head" staining pattern[11]. Two well-characterized antibodies yielding this immunohistochemistry pattern (anti-Yo and anti-Tr/DNER) were found negative in our patients. As a limitation of this study, we were unable to investigate other antibodies showing the same pattern. Antibodies with this staining pattern are associated with severe subacute cerebellar symptoms leading to prominent cerebellar atrophy and underlying cancer and have not been previously related with AIS. Since our patients did not exhibit

findings of apparent cerebellar involvement or systemic cancer, we did not consider the screening of other rarely detected paraneoplastic cerebellar antibodies[11].

Only one third of the AIS patients developed cerebellar antibodies. This might be a reflection of the different responsiveness of the immune system to the antigenic challenge. Putatively, AIS patients with antibodies might have genetic features leading to increased pro-inflammatory lymphocyte activation, enhanced antigen presentation, blood-brain barrier disruption or neuroinflammation. A notable finding in support of this argument was the increased prevalence of inflammation marker elevation in antibody positive patients, possibly indicating an immunogenetic background causing an overall propensity to antibody production.

Diffuse staining observed in granular and molecular layers of cerebellum is suggestive of cell surface antibodies that often have a pathogenic action[10], whereas the Medusa head pattern might be associated with both intracellular and cell surface antibodies[11]. However, there was no apparent difference in the overall severity of stroke in seronegative and seropositive patients and antibody-positive patients did not exhibit cerebellar signs. These results exclude a major pathogenic impact for anti-cerebellar antibodies and indicate that they are putatively directed against both intracellular and cell membrane antigens. An attractive hypothesis is that a mixed group of antibodies are produced in the weeks following stroke putatively as a bystander effect of the blood-brain barrier breach and increased access of the immune system to the brain. However, given the persistence of these antibodies, one may argue that long-term exposure to these antibodies might contribute more or less to post-stroke chronic disability. Unavailability of human brain tissue was a limitation of our study. Use of human brain tissue in future studies may provide a more useful perspective in terms of antibody binding and functionality of AIS-associated cerebellar antibodies.

In brief, why cerebellar antibodies are preferentially produced in AIS, whether these antibodies might contribute to overall long-term disability in AIS and whether our antibody assay may be used as a method to interpret the extent of post-stroke activation of the antigen-specific immune system need to be further studied. Our immediate plan for future exploration of cerebellar antibodies is to identify the target autoantigens using advanced molecular techniques including immunoprecipitation and mass spectrometry. Our second plan is to conduct long-term follow-up of antibody positive AIS patients with more extensive measures of cognitive and physical disability for more precise assessment of the long-term impact of cerebellar antibodies on stroke outcome.

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References

1. *Javidi E, Magnus T*. Autoimmunity after ischemic stroke and brain injury. Front Immunol 2019;10:686. https://doi.org/10.3389/fimmu.2019.00686
2. *Doyle KP, Quach LN, Solé M, Axtell RC, Nguyen TV, Soler-Llavina GJ, et al.* B-lymphocyte-mediated delayed cognitive impairment following stroke. J Neurosci 2015;35(5):2133-45. https://doi.org/10.1523/JNEUROSCI.4098-14.2015
3. *Sperber PS, Gebert P, Broersen LHA, Huo S, Piper SK, Teegen B, et al*. Serum anti-NMDA-receptor antibodies and cognitive function after ischemic stroke (PROSCIS-B). J Neurol 2022;269(10):5521-30. https://doi.org/10.1007/s00415-022-11203-x
4. *Kalev-Zylinska ML, Symes W, Little KC, Sun P, Wen D, Qiao L, et al*. Stroke patients develop antibodies that react with components of N-methyl-D-aspartate receptor subunit 1 in proportion to lesion size. Stroke 2013;44(8):2212-9. https://doi.org/10.1161/STROKEAHA.113.001235
5. *Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, et al*. Guidelines for the early management of patients with acute ischemic stroke: 2019 Update to the 2018 Guidelines for the early management of acute ischemic stroke: A guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2019;50(12):e344-e418. https://doi.org/10.1161/STR.0000000000000211
6. *Sansing LH, Tüzün E, Ko MW, Baccon J, Lynch DR, Dalmau J.* A patient with encephalitis associated with NMDA receptor antibodies. Nat Clin Pract Neurol 2007;3(5):291-6. https://doi.org/10.1038/ncpneuro0493
7. *Dambinova SA, Khounteev GA, Izykenova GA, Zavolokov IG, Ilyukhina AY, Skoromets AA*. Blood test detecting autoantibodies to N-methyl-D-aspartate neuroreceptors for evaluation of patients with transient ischemic attack and stroke. Clin Chem 2003;49(10):1752-62. https://doi.org/10.1373/49.10.1752
8. *Zerche M, Weissenborn K, Ott C, Dere E, Asif AR, Worthmann H, et al*. Preexisting serum autoantibodies against the NMDAR subunit NR1 modulate evolution of lesion size in acute ischemic stroke. Stroke 2015;46(5):1180-6. https://doi.org/10.1161/STROKEAHA.114.008323
9. *Royl G, Fokou TJ, Chunder R, Isa R, Münte TF, Wandinger KP, et al.* Antibodies against neural antigens in patients with acute stroke: joint results of three independent cohort studies. J Neurol 2019;266(11):2772-9. https://doi.org/10.1007/s00415-019-09470-2
10. *Hara M, Martinez-Hernandez E, Ariño H, Armangué T, Spatola M, Petit-Pedrol M, et al*. Clinical and pathogenic significance of IgG, IgA, and IgM antibodies against the NMDA receptor. Neurology 2018;90(16):e1386-e1394. https://doi.org/10.1212/WNL.0000000000005329
11. *Jarius S, Wildemann B*. Medusa head ataxia: the expanding spectrum of Purkinje cell antibodies in autoimmune cerebellar ataxia. Part 3: Anti-Yo/CDR2, anti-Nb/AP3B2, PCA-2, anti-Tr/DNER, other antibodies, diagnostic pitfalls, summary and outlook. J Neuroinflammation 2015;12:168. https://doi.org/10.1186/s12974-015-0358-9