Plasma Amyloid-Beta Peptides in Acute Cerebral Ischemia: A Pilot Study

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Background: Blood-based tests for a rapid and valid diagnosis as well as outcome prognosis of acute stroke are desirable. Recently, plasma A β 40 was suggested as an independent cerebrovascular risk factor candidate. **Methods:** We investigated eight plasma samples of patients with clinical signs of acute cerebral ischemia for derangements of plasma amyloid-beta (A β) peptide patterns as compared to 13 patients with other neuropsychiatric diseases. For the analysis of plasma, we used immunoprecipitation followed by the quantitative A β -SDS-PAGE/immunoblot. **Results:** The ma-

jor outcome was a striking decrease of A β 1–40 in plasma paralleled by an increase in the ratio of A β 1–38/A β 1–40 in two patients with acute stroke. Interestingly, these patients had an onset of symptoms within only 2–4 hr before venous puncture and there was a strong correlation of A β 1–38/A β 1–40 levels with the time span between onset of symptoms and venous puncture. **Conclusion:** From these results, we suggest the ratio of plasma A β 1–38/A β 1–40 as a possible biomarker for the early diagnosis of acute stroke. J. Clin. Lab. Anal. 26:238–245, 2012. © 2012 Wiley Periodicals, Inc.

Key words: acute cerebral ischemia; biomarkers; amyloid- β peptides

INTRODUCTION

Ischemic stroke is a leading cause of death and disability in developed countries (1) and its clinical differential diagnosis is usually a matter of urgency. There is an intense search for biomarkers that may aid the early diagnosis and prognosis of stroke or differentially judge its cause (2). In case of thromboembolia, the decision for or against thrombolysis depends on the duration of deficits and the estimated outcome prognosis. Thus, a biomarker with plasma concentrations, depending on the extent of the ischemic brain area and the duration of cerebral ischemia, may be useful in clinical practice. As the dimension of infarcted brain area and the duration of cerebral ischemia often crucially determine the clinical outcome in the long run, such a marker may be suited for the prognosis of stroke, as well.

We identified the same quintet of A β peptides, i.e., A β 1–37/38/39/40/42, which our group had characterized as regular constituents of human cerebrospinal fluid (CSF)

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(3), to also occur in plasma (4). However, plasma A β peptides were characterized by the additional aminoterminally truncated species A β 2–40. Plasma A β peptides have been investigated for their diagnostic value in differential and early diagnosis of Alzheimer's disease (5) and we have observed disease-specific derangements in patients with vascular dementias (6). Furthermore, another publication demonstrated a correlation between plasma A β 40 concentration as measured by ELISA and the level of vascular injuries in mild cognitive impairment (MCI), Alzheimer's disease (AD), and cerebral amyloid angiopathy (CAA) (7).

Previously published data on CSF A β peptides indicate that the relative amounts of A β peptides reflect disease-specific derangements of A β peptide homeostasis more adequately as compared to the absolute amounts of single A β peptide species (3, 8). Accordingly, a focus of the present investigation is to study the impact of acute cerebral ischemia (ACI) on the A β -peptide blood signature and not only on single A β peptide species.

METHODS

Participants

We investigated 21 plasma samples referred to our laboratory between 2006 and 2007. Plasma samples of patients with stroke and neuropsychiatric disorders, respectively, came from patients of the University of Göttingen.

A psychiatrist and a neurologist rendered diagnoses based on thorough anamnesis, clinical examination, clinical records, and best clinical judgement. These investigators were blinded to the neurochemical outcome measures. Investigations were carried out with the informed consent of patients or their authorized caregiver. The study was conducted under the guidelines of the Declaration of Helsinki (9) and approved by the ethics committee of the University of Göttingen.

Neuropsychiatric disorders (ND)

The 13 patients with ND underwent diagnostic venipuncture during routine clinical workup. The following diagnoses were made according to the criteria of DSM-IV: epilepsy ($n = 2$), tension headache ($n = 3$), polyneuropathy ($n = 2$), depression ($n = 1$), schizophrenia ($n = 1$), multiple sclerosis ($n = 1$), alcohol dependence ($n = 1$), Muenchhausen's syndrome ($n = 1$), and subcortical arteriosclerotic encephalopathy ($n = 1$). Patients with the suspicion of ACI or dementia were excluded.

Patients with ACI

Diagnosis of an acute cerebrovascular ischemic event (i.e., an ischemic stroke or a transient ischemic attack

TABLE 1. Absolute and percentage abundances of plasma A β peptide species and demographic data of the diagnostic groups

Diagnosis	ND ($n = 13$) Mean	ND ($n = 13$) $\pm$ SD	ACI ($n = 8$) Mean	ACI ($n = 8$) $\pm$ SD
Age	47.5	17.1	81.0	11.6
Time lag (h)	—	—	7.42	3.83
A β 1–37 (ng/ml)	0.011	0.003	0.013	0.005
A β 1–38 (ng/ml)	0.017	0.003	0.018	0.007
A β 1–39 (ng/ml)	0.019	0.004	0.020	0.009
A β 1–40 (ng/ml)	0.155	0.025	0.125	0.073
A β 1–42 (ng/ml)	0.018	0.008	0.014	0.005
A β 2–40 (ng/ml)	0.013	0.004	0.011	0.005
total A β (ng/ml) ^a	0.233	0.040	0.200	0.095
A β 1–37% ^b	4.577	0.791	7.994	4.287
A β 1–38% ^b	7.316	1.294	10.512	4.273
A β 1–39% ^b	8.206	1.314	10.424	3.823
A β 1–40% ^b	66.860	2.977	56.795	16.156
A β 1–42% ^b	7.572	2.209	8.283	3.929
A β 2–40% ^b	5.468	1.167	5.992	2.092
Ratio A β 1–38/1–40	0.110	0.022	0.238	0.218
Ratio A β 1–37/1–40	0.069	0.014	0.190	0.208

^aTotal A β -peptide concentration as measured by the sum of all investigated A β peptides;

^bPercentage abundance of A β peptides relative to the sum of all investigated A β peptides.

(TIA)) was rendered in eight patients based on the standard clinical definition (10). All included patients received neuroimaging (i.e., computerized tomography (CT) or magnetic resonance imaging (MRI)). The time lag from first onset of symptoms to the time of venous puncture was available for six of eight patients and ranged from 2 to 12 hr. Retrospective assortment to Trial of Org 10172 Acute Stroke Treatment (TOAST) criteria (11) revealed one patient within group 1, two patients within group 2, two patients within group 3, and three patients within group 5. Table 1 shows the patients' characteristics of each patient group. Table 2 displays the baseline characteristics of investigated patients.

Test Methods

Plasma from patients with ACI showing prevalent focal neurological deficits was drawn by venous puncture at the time of arrival in the emergency unit. Plasma of patients with ND was drawn for routine laboratory assessments during the time of hospitalization. Plasma was drawn into EDTA tubes and sampled in polypropylene vials and centrifuged (1,000 g , 10 min, and 4°C). The preanalytical handling of samples followed a standardized protocol according to previously published data (4). Samples were kept frozen before analysis for a maximum of 6 month.

TABLE 2. Baseline characteristics of investigated patients

Diagnostic group	No.	Vascular risk factors	Overt vascular diseases	Medical conditions	Medications used
ACI	1	Arterial hypertension	Coronary heart disease	Absolute arrhythmia	Aspirin, Furosemide, Digoxin, Isosorbiddinitrate, Verapamil
ACI	2	Arterial hypertension, hypercholesterolemia	—	Schizophrenia	Benperidol, Biperiden
ACI	3	Arterial hypertension	—	Absolute arrhythmia	Aspirin, Digitoxin, Verapamil, Enalapril, Furosemide
ACI	4	Arterial hypertension	Subcortical arteriosclerotic encephalopathy	Absolute arrhythmia	Lisinopril, Molsidomine, Verapamil, Phenprocoumon
ACI	5	Arterial hypertension	Subcortical arteriosclerotic encephalopathy,	—	Aspirin, Lisinopril, Hydrochlorothiazide, Torasemide
ACI	6	Arterial hypertension	Subcortical arteriosclerotic encephalopathy	Atrioventricular block, aortic valve stenosis	Esomeprazole, Enalapril, Xipamide, Metamizol
ACI	7	Arterial hypertension, hyperlipidemia, diabetes mellitus	Subcortical arteriosclerotic encephalopathy	Focal epilepsy	Aspirin, Enalapril, Simvastatin, Verapamil, Hydrochlorothiazide, Terazosin, Carbamazepine, Metformin
ACI	8	Arterial hypertension, hypercholesterolemia, diabetes mellitus	—	Vertebral disc herniation	Ramipril, Hydrochlorothiazide, Diclofenac, Metamizol, Pantoprazole, Amitriptyline, Bromazepam, Insulin
ND	9	—	—	Muenchhausen's syndrome, focal epilepsy, bronchial asthma	Lamotrigine, Valproate
ND	10	Arterial hypertension	—	Depression, nephrolithiasis	Metoprolol, Amlodipine, Hydrochlorothiazide, Sertraline, Amitriptyline, Reboxetine, Pipamperone
ND	11	—	—	Focal epilepsy	Valproate
ND	12	Arterial hypertension, hyperhomocysteinemia, diabetes mellitus	Subcortical arteriosclerotic encephalopathy	Atrial thrombosis	Insulin, Phenprocoumon
ND	13	Smoking	—	Multiple Sclerosis, hypothyroidism	Methylprednisolone, Mitoxantrone, Levothyroxine
ND	14	—	—	Schizophrenia	Clozapine, Pantoprazol,

TABLE 2. Continued

Diagnostic group	No.	Vascular risk factors	Overt vascular diseases	Medical conditions	Medications used
ND	15	—	—	Alcohol dependence, alcohol hallucinosis	Olanzapine, Melperone
ND	16	Arterial hypertension, diabetes mellitus	—	Polyneuropathy, hypothyroidism	Levothyroxine, Candesartan, Hydrochlorothiazide, Diclofenac, Mirtazapine, Pantoprazole, Pregabalin
ND	17	Arterial hypertension	—	Polyneuropathy, Restless legs syndrome, myelodysplastic syndrome	Bisoprolol, Trimipramine, Maprotiline, Tilidine, Naloxone
ND	18	—	—	Focal epilepsy	Levetiracetam, Lamotrigine, Amitriptyline
ND	19–21	—	—	Tension headache	—

Two experienced technical assistants blinded to the clinical diagnosis performed all neurochemical measurements and quantifications.

Immunoprecipitation and A β -SDS-PAGE/immunoblot of plasma

For immunoprecipitation, magnetic sheep anti-mouse IgG Dynabeads M-280 (Dyna, Hamburg, Germany) were incubated overnight at +4°C with the monoclonal A β amino terminally selective antibody, 1E8 (provided by Schering, Berlin, Germany) according to the manufacturer's protocol. Five hundred microliter of plasma were added to 200 μ L of five-fold concentrated Radioimmuno-precipitation assay buffer (RIPA) detergent buffer (3), 25 μ L of the activated magnetic Dynabeads (1 μ g monoclonal Antibody 1E8/ 1.68×10^7 beads), and 300 μ L of H₂O. Samples were then incubated overnight at +4°C under rotation, following the washing of the beads four times with PBS/0.1% of bovine serum albumin and once with 10 mM Tris/HCl, pH 7.4. For A β -SDS-PAGE/immunoblot, bound A β peptides were eluted by heating the samples to 95°C for 5 min with 25 μ L of SDS-PAGE sample buffer (3).

For the separation of A β peptides, a modified urea version of the Bicine-/bis-Tris/Tris/sulfate SDS-PAGE was used as described by Wiltfang et al. (3). The composition of the separation gel was modified from 12%T to 10%T. Briefly, immunoprecipitated plasma (8 μ L of immunoprecipitate) in SDS-PAGE sample buffer was applied to a gel slot. After running the gels at a constant current of 24 mA per gel at room temperature for 1 hr, semidry Western blotting was performed according to the proto-

col of Wiltfang et al. (3). For the immunological detection of A β peptides on PVDF-membranes, the amino terminally selective mouse monoclonal antibody 1E8 (provided by Schering, Berlin, Germany; stock: 0.25 mg/ml) was diluted 4,000-fold and applied overnight at 4°C. Membranes were further incubated with a biotinylated anti-mouse polyclonal antibody (Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase coupled streptavidin (Amersham Pharmacia Biotech, Buckinghamshire, England) for 1 hr each. Washing steps were performed in between and chemiluminescent visualization was done by ECL-Plus solution (Amersham Pharmacia).

Plasma samples and peptide standards were run on the same gel as triplicates at minimum. Each gel carried a five-point dilution series of synthetic A β peptides A β 1–37, A β 1–38, A β 1–39, A β 1–40, and A β 1–42. A β 2–40 was only applied in the two lower concentrations, because the bands of A β 2–40 and A β 1–42 tended to diffuse into one single band when higher concentrations were applied. Thus, the three higher concentrations of dilution series of A β 1–42 were used to complement the standard row of A β 2–40. Synthetic peptides A β 1–38, A β 1–40, A β 1–42, and A β 2–40 were obtained from Bachem (Bubendorf, Switzerland) and A β 1–37 and A β 1–39 were synthesized automatically according to Janek et al. (12). Standard preparations of synthetic A β peptide mixture were created as described previously (13) and bands were quantified from individual blots of each patient relative to this dilution series using a charge-coupled device camera (CCD-camera) and Quantity-ONE software (FluorSMax MultiImager, Bio-Rad).

The detection sensitivity of the A β -SDS-PAGE/immunoblot was 0.6–1 pg (3, 13). Coefficients

of variation of the A β peptides' concentrations ranged between 14.0% and 18.5% (4).

Statistical Analysis

Patient groups were characterized by mean deviation and standard deviation. Plasma peptide values are given in absolute (pg/ml) or percentage (amount of each peptide relative to the sum of all single investigated A β peptides) values, respectively. Significant group differences were evaluated with the Mann-Whitney U-test. Comparisons of multiple groups or parameters were not performed. Spearman's rho was used for correlation analysis. The two-sided level of significance was taken as $P < 0.05$. The statistical software package SPSS, versions 10.0 and 12.0 served for computations.

RESULTS

Test Results

Group differences

The ND group was significantly younger than the ACI group ($P < 0.01$). There was no correlation of age with any of the analyzed parameters in the patient groups. The groups did not vary significantly in the absolute concentrations of any of the measured A β peptides. In percentage terms (A β 1-X%), the ACI group presented with higher levels of A β 1-37 ($P = 3.4 \times 10^{-3}$) and A β 1-38 ($P = 2.0 \times 10^{-2}$). Accordingly, the ratios of A β 1-37/A β 1-40 ($P = 7.7 \times 10^{-3}$) and A β 1-38/A β 1-40 ($P = 2.0 \times 10^{-2}$) were significantly higher in ACI as compared to ND (Figures 1 and 2).

Plasma A β peptides in ACI

The aforementioned significant group differences disappeared for A β 1-38 and A β 1-38/A β 1-40, when two patients with stroke who displayed very low levels of A β 1-40, accompanied by a pronounced increase of A β 1-38 and A β 1-37, respectively, were omitted from the ACI group. A β 1-37% levels ($P = 4.6 \times 10^{-2}$) and the ratio A β 1-37/A β 1-40 ($P = 4.6 \times 10^{-2}$) were still elevated, but to a lesser degree. These two patients displayed percentage levels of A β 1-38 and A β 1-40, respectively, differing more than 1 SD from the mean of the ACI group (Figure 3). Interestingly, we found a highly significant positive correlation when the estimated time span between the first onset of focal neurological symptoms and venous puncture (time lag) was compared to A β 1-40% levels (Spearman's Rho = 0.926, $P = 7.9 \times 10^{-3}$) (Figure 4). Conversely, A β 1-37% ($P = 2.1 \times 10^{-2}$) and A β 1-38% ($P = 1.2 \times 10^{-2}$) levels were negatively correlated with the time lag. The Spearman correlation for A β 1-38/A β 1-40 with the

Fig. 1. Scatterplots and means of the ratio A β 1-38/A β 1-40 in the plasma of ND and ACI.

Fig. 2. Scatterplots and means of the ratio A β 1-37/A β 1-40 in the plasma of ND and ACI.

time lag was -0.943 ($P = 4.8 \times 10^{-3}$). However, the latter data were only available for six of eight patients. In two patients, the onset of symptoms could not be estimated due to insufficient data in the medical history, but symptoms were still acute/recent at the time of venous puncture.

Fig. 3. Scatterplot of ND and ACI divided by plasma Aβ1-38% and Aβ1-40%.

Fig. 4. Linear correlation of time lag between first onset of neurological symptoms and Aβ1-40% (Spearman's $Rho = 0.926$, $p = 7.9 \times 10^{-3}$) in six ACI patients.

Discussion

Herein, we present a detailed characterization of Aβ peptide species in the plasma of patients with symptoms of ACI in comparison to other ND without signs of ACI. The major outcome was that ACI displayed significantly higher levels of Aβ1-37%, Aβ1-38%, accompanied by decreased Aβ1-40% plasma levels in the sense of a disease-specific plasma-based Aβ peptide fingerprint. Otherwise, a scatterplot analysis revealed that this pattern predominantly occurred in two patients, who experienced the onset of symptoms of cerebral ischemia 2–4 hr before venous puncture, respectively. At the time of clinical investigation and venous puncture, signs of brain infarction were still absent in CT in these two patients. Both patients had clin-

ical signs of a partial infarction of the left medial cerebral artery. One of the two patients had persistent neurological deficits for at least 4 days and a follow-up CT revealed a left temporal hypodense lesion. The other patient recovered within 24 hr from neurological deficits and did not receive a follow-up CT. These results indicate that the underlying plasma Aβ-peptide derangements seem to take place during a very short time span after the onset of clinical symptoms of cerebral ischemia. Accordingly, a correlation analysis revealed a strong correlation of the levels of plasma Aβ1-40% with the estimated time span between the first onset of focal neurological deficits and venous puncture (time lag) in ACI. Taken together, these findings suggest a close relationship between plasma Aβ1-37%, Aβ1-38% and Aβ1-40% levels and the time course of ischemic brain disorders.

Possible underlying pathogenic mechanisms

We can only speculate on the underlying pathogenic mechanisms of our findings. It has been reported that a certain domain of the β-amyloid precursor protein (βAPP) that inhibits proteases (Kunitz protease inhibitor domain, KPI) is closely involved in blood homeostasis. For example, in a mouse model of artificial brain hemorrhage, the impact of over and under expression of KPI-containing βAPP isoforms on the extension of cerebral hemorrhage was examined. The main outcome was that mice constructed to over express the KPI-containing βAPP and mice with blocked expression of KPI-containing βAPP had larger and smaller hemorrhages than wild-type mice, respectively (14, 15). It is also known from other studies that KPI-containing protease inhibitors are capable of blocking certain steps of blood coagulation such as thrombin formation (16, 17). These results indicate that the KPI domain of βAPP may play a role in the regulation of blood clotting. Notably, cerebral hemorrhage occurs in up to 65% of the affected brain regions and deposits of Aβ have been found inconsistently in cerebral blood vessels in the course of ischemic stroke (18). In this coherency, it has also been suggested that Aβ peptides themselves might be employed for inhibiting thrombosis and thereby promote hemorrhagic complications of ischemic stroke (18). Given this, one mechanism of the decreasing amount of plasma Aβ1-40 after cerebral ischemia may be its downregulated production under conditions in which large amounts of βAPP may be needed in terms of anticoagulation and therefore do not undergo its normal metabolism to produce Aβ-peptide species. Another possibility is that Aβ1-40 itself may be involved in thrombolysis during cerebral ischemia, thereby adhering to blood vessel walls, which may lead to a reduction of its soluble content in plasma.

Otherwise, we found reverse plasma Aβ peptide derangements (i.e., high Aβ1-40 and low Aβ1-38 levels)

in patients with vascular dementias as compared to nondemented controls (6). These findings are in line with the elevation of plasma A β 1–40 in the diffuse subtype of small vessel disease (19) and its positive correlation to the extension of white matter lesions in MRI studies (7, 20). It has been proposed that these observations may be related to the pathophysiological role of A β 1–40 in disrupting endothelial vascular function (19). From their results, Gurol et al. (7) reported A β 40 to be a potential cerebrovascular risk factor. Beside this and in light of the above considerations, the high values of A β 1–40 in these studies may also be interpreted as an epiphenomenon of upregulated anticoagulating factors in order to counteract a chronic affinity toward hemostasis.

Taken together, the discussed results support the hypothesis that either β APP or A β peptides or both may interact with blood homeostasis. Although the underlying mechanisms remain to be clarified, A β 1–40 plasma levels may quite directly reflect changes of the β APP metabolism during different states of activity in blood homeostasis.

Possible implications with regard to biomarker aspects

Our findings may have implications for the blood biomarker-based monitoring of stroke and related cerebrovascular disorders. With regard to stroke, A β 1–40 may be useful in order to specify the clinical estimation of the duration of hemostatic disorder in the brain and the individual risk of hemorrhagic complications. As the drop of plasma A β 1–40 seems to be a short-term effect after stroke, the concentration of plasma A β 1–40 may tell the clinical investigator about the onset of cerebral ischemia. Moreover, the plasma A β 1–40 concentration may be related to the risk of complicating hemorrhages during ischemic stroke as discussed above. Both aspects are highly important in predicting the successfulness of therapeutic thrombolysis, and further studies are required to evaluate our findings in this direction.

In chronic cerebrovascular disorders, high plasma A β 1–40 levels may represent a surrogate marker of endothelial dysfunction, but may also be an expression of a continuous countersteering against hypercoagulation. In the latter case, A β 1–40 levels may be of interest for the monitoring of sufficient anticoagulative therapeutics.

Limitations of the study and future prospects

The reliance on clinical diagnosis limits our results because of potential misclassification. In this coherency, further studies that include more homogenous groups of patients and controls are needed. Another point of concern is the size of patient groups, especially in light of the fact that only two patients in the group of ACI displayed a dis-

tinctive pattern of plasma A β peptides. Retrospectively, the broad time range of up to 12 hr between onset of symptoms and blood sampling seems to have considerably accounted for the variability of values in the small group of ACI patients. Under this aspect, the investigation of plasma A β peptide levels within shorter time spans after the onset of symptoms is currently under preparation.

The possible diagnostic meaning of plasma A β peptide patterns may be most suitably evaluated in longitudinal population based studies with long monitoring periods and closely meshed monitoring of plasma β APP and A β -peptide levels, respectively, during ACI. In this context, a year-long clinical follow up may be helpful to clarify whether plasma A β peptide levels may even support the prognosis after cerebral ischemia.

The A β -SDS-PAGE/immunoblot can be considered as a time-consuming and low-throughput method. Its results may rather help to find new biomarker candidates than to serve for routine analysis. Thus, for diagnostic purpose, possible biomarker candidates will have to be integrated in conventional assay formats (e.g., ELISA, multiplex platforms), especially when considering stroke as a case of emergency.

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