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Research Articles

Deficiency of fibrinolytic enzyme activities in the serum of patients with Alzheimer-type dementia

T. Aoyagi[a, b], T. Wada[a], F. Kojima[a], M. Nagai[a], S. Harada[a], T. Takeuchi[a], K. Isse[c], M. Ogura[c], M. Hamamoto[c], K. Tanaka[c] and T. Nagao[c]

[a] *Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141 (Japan),* [b] *Showa College of Pharmaceutical Sciences, 3-chome, Higashitamagawa-gakuen, Machida-shi, Tokyo 194 (Japan), and* [c] *Tokyo Metropolitan Tama Geriatric Hospital, 1-7-1 Aoba-cho, Higashi-Murayama-shi, Tokyo 187 (Japan)*
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Abstract. Previously we reported that there is a kallikrein deficiency in the cerebral tissue of patients with Alzheimer-type dementia. The present study was performed to investigate protease changes in the serum of these patients. The results showed that the kallikrein activity was normal, but that the activities of plasmin and urokinase were significantly low. The present findings indicate a derangement in the clotting and fibrinolytic systems in Alzheimer patients.
Key words. Alzheimer's disease; patients' serum; clotting system; fibrinolytic system; plasmin; urokinase; thrombin.

A number of studies have indicated that the accumulation of abnormal proteins in the brain is pathogenetically related to Alzheimer's disease[1–4]. Injection of a protease inhibitor into animal brains was shown to induce the formation of lysosome-associated granular aggregates (dense bodies) which closely resembled the ceroid-lipofuscin that accumulates in certain disease states and in the process of aging[5]. This indicated that abnormal protease activities may play an important role in the development of Alzheimer's disease. In agreement with this hypothesis, we previously found kallikrein deficiency in the cerebral tissues of the patients with Alzheimer-type dementia[6].

In addition to the changes in the cerebral tissues, many biochemical changes have been reported in the peripheral tissues of patients with this disease[7]. They include altered membrane fluidity of platelets[8], increased X-ray sensitivity of fibroblasts[9], reduced free Ca^{++} in fibroblasts[10], reduced fibroblast spreading[11], reduced lymphocyte acetylcholine esterase activity[12], reduced secretion of cholinergic neuron differentiation factor from fibroblasts[13], and decreased adhesiveness of fibroblasts[14].

Table 1. List of the proteases measured and their substrates

Enzyme	Abbreviation	Substrate	Reference for assay method
Aspartate aminopeptidase (EC 3.4.11.7)	AP-A	Glu · NA	17
Arginine aminopeptidase (EC 3.4.11.6)	AP-B	Arg · NA	17
Leucine aminopeptidase (EC 3.4.11.1)	Leu-AP	Leu · NA	17
Dipeptidyl peptidase II (EC 3.4.14.2)	DPP-II	Lys-Ala · NA	18
Dipeptidyl peptidase III (EC 3.4.14.4)	DPP-III	Arg-Arg · NA	19
Dipeptidyl peptidase IV (EC 3.4.14.5)	DPP-IV	Gly-Pro · NA	20
Arginine carboxypeptidase (EC 3.4.17.3)	CP-N	Hip-Lys	21
Angiotensin-converting enzyme (EC 3.4.15.1)	ACE	Hip-His-Leu	21
Cathepsin B (EC 3.4.22.1)	Cathepsin B	Z-Arg-Arg · NA	22
Plasma kallikrein (EC 3.4.21.34)	Kallikrein	Pro-Phe-Arg · MCA	23
Plasmin (EC 3.4.21.7)	Plasmin	Boc-Val-Leu-Lys · MCA	24
Urokinase (EC 3.4.21.31)	UK	Glt-Gly-Arg · MCA	24
α-Thrombin (EC 3.4.21.5)	Thrombin	Boc-Val-Pro-Arg · MCA	25
Lymphoid serine protease	LSP	Z · Lys · S-Bzl	26

Abbreviations used: Glu · NA, L-glutamic acid β-naphthylamide hydrochloride; Arg · NA, L-arginine β-naphthylamide hydrochloride; Leu · NA, L-leucine β-naphthylamide hydrochloride; Lys-Ala · NA, L-lysyl-L-alanine β-naphthylamide; Arg-Arg · NA, L-arginyl-L-arginine β-naphthylamide; Gly-Pro · NA, glycyl-L-proline β-naphthylamide; Hip-Lys, hippuryl-L-lysine; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; Z-Arg-Arg · NA, benzyloxycarbonylarginyl-L-arginine β-naphthylamide; Pro-Phe-Arg · MCA, L-prolyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide; Boc-Val-Leu-Lys · MCA, t-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4 methylcoumaryl-7-amide; Glt-Gly-Arg · MCA, glutaryl-glycyl-L-arginine 4-methylcoumaryl-7-amide; Boc-Val-Pro-Arg · MCA, t-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine 4-methylcoumaryl-7-amide; Z-Lys · S-Bzl, N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester.

These reports prompted us to investigate whether the systemic changes that occur in Alzheimer patients include changes in serum proteases.

Materials and methods

Sera of control subjects and patients with dementia. We selected three age-matched groups: 11 cases of Alzheimer-type dementia (primary degenerative dementia of the Alzheimer-type, 75.2 ± 2.72 years old; mean ± standard error), the same number patients with vascular dementia (74.6 ± 2.18 years old) and 23 control subjects (75.1 ± 1.44 years old). Alzheimer-type dementia was diagnosed by *DSM-III-R* criteria[15], and vascular dementia was diagnosed by *NINDS* criteria[16]. Blood samples were collected for examination before breakfast in the morning.

Determination of enzyme activities. The substrates and enzymes, and their sources, were as follows (see table 1 for meaning of abbreviations)[12,17–25]; Glu · NA, Arg · NA, Leu · NA, Lys-Ala · NA, Arg-Arg · NA and Gly-Pro · NA from Bachem Feinchemikalien, AG, Bubendorf, Switzerland; Hip-Lys, Hip-His-Leu, Pro-Phe-Arg · MCA, Boc-Val-Leu-Lys · MCA, Glt-Gly-Arg · MCA, Boc-Val-Pro-Arg · MCA and Z · Lys · S-Bzl from Peptide Institute Inc, Osaka, Japan. Z-Arg-Arg · NA was synthesized in our laboratory.

The serum was dispensed into microwell plates (nunclone, F96) for aminopeptidases and into test tubes (1.5 × 10 cm) for endopeptidases, and the appropriate substrates added. Incubation was carried out for 1 h at 37 °C. For the aminopeptidase (AP) and cathepsin B assays, 50 µl of 2.5 mM β-naphthylamide derivative was used as the substrate, and the absorbance at 525 nm was determined using a microplate reader model 3550 (BIO-RAD)[17–20,22]. For the carboxypeptidase assay the buffers used were 0.05 M Tris-HCl buffer (pH 8.0) for CP-N and 0.05 M Tris-HCl containing 0.03 M NaCl (pH 8.0) for ACE, to which the respective substrates were added[12]. For the endopeptidase assay, 20 µl of 2.5 mM 4-methylcoumaryl-7-amide derivative was used and the fluorescence spectrum (EX 380 nm, Em 460 nm) was determined by the HITACHI MDF-4 fluorimeter[23–26].

The references for the assay methods and substrates used are listed in table 1. All the enzyme assays were done in triplicate, and their standard deviations were within 10% of the average values[12]. The units of enzyme activities were expressed as nmols of reaction products generated during 1 min of incubation per ml of serum. Protein was determined by the method of Lowry et al.[27].

The time-courses of the enzyme reactions were linear for at least 60 min and the enzymatic activities were linear with respect to the serum volume in the assay medium[17]. The synthetic substrates used for the assay of endopeptidase activities can be cleaved by miscellaneous enzymes different from the target enzymes. In order to exclude such nonspecific effects, we utilized the inhibitor bestatin[6]. By this means we were able to achieve linearity in the endopeptidase assays.

Statistical analysis. Comparisons among the three groups were made with the analysis of variance (ANOVA). The enzyme levels found to be significantly different with ANOVA were further analyzed with Bonferroni's method for differentiation between two groups. The computations were done with a statistical software distributed by Kyoritu Shuppan Co., Tokyo[28].

Results and discussion

In table 2 the serum levels of proteases are compared between three groups: the control subjects, the patients with vascular dementia, and the patients with Alzheimer-type dementia.

Table 2. Enzymatic changes in serum of patients with dementia

Enzyme	Specific activity ± SE (nmol/min/ml) Control (n = 23)	Vascular (n = 11)	Alzheimer (n = 11)	ANOVA
AP-A	5.34 ± 0.29	5.23 ± 0.44	4.43 ± 0.23	NS
AP-B	14.69 ± 1.22	15.21 ± 1.55	14.44 ± 0.67	NS
Leu-AP	27.20 ± 2.58	27.67 ± 2.94	23.68 ± 1.25	NS
DPP-II	1.89 ± 0.21	1.97 ± 0.16	2.00 ± 0.16	NS
DPP-III	0.69 ± 0.11	0.94 ± 0.12	1.10 ± 0.13	NS
DPP-IV	16.01 ± 1.26	14.20 ± 1.63	17.00 ± 1.26	NS
CP-N	166.68 ± 6.06	158.34 ± 9.77	156.74 ± 8.40	NS
ACE	3.58 ± 0.27	3.19 ± 0.53	3.56 ± 0.32	NS
Cathepsin B	0.16 ± 0.03	0.11 ± 0.04	0.16 ± 0.03	NS
Kallikrein	111.46 ± 12.13	95.04 ± 7.63	123.18 ± 5.91	NS
Plasmin	0.76 ± 0.03	0.66 ± 0.06	0.53 ± 0.03	$p < 0.01$
Urokinase	0.40 ± 0.03	0.36 ± 0.03	0.28 ± 0.04	$p < 0.05$
Thrombin	153.14 ± 15.66	130.57 ± 24.09	105.19 ± 17.08	NS
LSP	41.57 ± 1.91	41.85 ± 2.72	43.15 ± 1.82	NS

SE: Standard error of mean, NS: not significant.

When tested with ANOVA, only plasmin and urokinase showed significant differences among the three age-matched groups. In order to examine the difference in more detail we used Bonferroni's method. As can be seen in table 3, both plasmin and urokinase showed significant differences only between the control and the Alzheimer groups.

The present results apparently showed the difference between the two types of dementia: Alzheimer-type dementia and that caused by cerebrovascular disease. The results were contrary to our expectations in that the former, rather than the latter, was more closely related to abnormality in the fibrinolytic and clotting system. Although these results may seem paradoxical, we must note that there are several reports suggesting the relationship between Alzheimer's disease and the clotting system [8, 9]. Alzheimer's disease is characterized by extracellular deposits of amyloid β/A4 protein fibrils in senile plaque cores and in vessel walls in the brain. Amyloid beta-protein precursor (APP) represents a family of transmembrane glycoproteins containing amyloid beta-protein, and part of this protein is included in the transmembrane sequence. Cole et al. reported that stimulated platelets release membrane fragments containing full-length C-terminal and N-terminal immunoreactive APP which should contain an intact β/A4 sequence [29]. They confirmed that human platelets can be stimulated by thrombin (or ionomycin) to secrete soluble truncated APP and particulate membrane fragments which contain C-terminal and N-terminal immunoreactive APP.

Table 3. Results of analysis with Bonferroni's method

		Group difference		
		Control	Vascular	Alzheimer
Plasmin	Control	–	NS	$p < 0.01$
	Vascular	NS	–	NS
		Group difference		
		Control	Vascular	Alzheimer
Urkinase	Control	–	NS	$p < 0.05$
	Vascular	NS	–	–NS

NS: not significant.

At present we do not have direct evidence relating these previous observations to the present results. Nevertheless, it is possible that in this disease there are some metabolic abnormalities which are related, on the one hand, to the abnormal processing of the protein precursor and, on the other, to a disequilibrium between thrombin and plasmin or urokinase, the levels of which were found in the present study to be changed. Finally, if the present observations have a pathophysiological meaning, one would expect that the administration of tissue plasminogen activator or urokinase would have some beneficial effects in patients with Alzheimer-type dementia. More studies are needed to confirm this hypothesis.

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Effects of some GABAergic agents on quinine-induced seizures in mice

G. J. Amabeoku and O. Chikuni

Department of Clinical Pharmacology, Medical School, University of Zimbabwe, P.O. Box A178, Avondale, Harare (Zimbabwe)
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Abstract. The effects of some GABAergic agents on seizures induced by quinine were studied in mice. Muscimol, AOAA, DABA and baclofen significantly protected mice against quinine-induced convulsions. Bicuculline effectively enhanced quinine-induced convulsions, and significantly attenuated the protective effects of muscimol, AOAA and DABA against convulsions induced by quinine. Diazepam and phenobarbitone significantly protected mice against convulsions induced by quinine. However, phenytoin did not affect quinine-induced seizures to any significant degree. These results indicate that the convulsant effect of quinine may be due to a disturbance in the status of the GABAergic system.
Key words. Quinine; seizures; GABAergic agents; GABAergic mechanism; diazepam; phenobarbitone; phenytoin.

Quinine, a cinchona alkaloid, is very consistent and effective in the treatment of severe malaria caused by chloroquine-resistant *Plasmodium falciparum* [1–4]. However, quinine in toxic doses can cause convulsions in patients [5]. The mechanism of the induction of seizures by antimalarial drugs is uncertain [6]. Since GABA is the major inhibitory neurotransmitter in the mammalian nervous system, impairment of GABA-mediated neurotransmission is a major factor underlying epileptic phenomena [7, 8]. The purpose of this study is, therefore, to investigate the influence of muscimol, aminooxyacetic acid (AOAA), diaminobutyric acid (DABA), baclofen, bicuculline, diazepam, phenobarbitone and phenytoin on quinine-induced seizures in mice, with a view to elucidating the mechanism underlying quinine seizures.

Materials and methods

Animals. Male albino mice (inbred in our Animal House, University of Zimbabwe, Harare) weighing 20–30 g were used in the study. The mice were normally housed in groups of eight per cage and maintained on tap water and food ad libitum. Each mouse was used for one experiment only.

Drugs. The following drugs were used: quinine hydrochloride (Sigma Chemical Co.), muscimol (Sigma Chemical Co.), aminooxyacetic acid hemihydrochloride (AOAA, Sigma Chemical Co.), DL-2,4-diamino-n-butyric acid dihydrochloride (DABA, Sigma Chemical Co.), baclofen (Sigma Chemical Co.), 5,5-diphenylhydantoin sodium salt (Phenytoin, Sigma Chemical Co.) and phenobarbitone (Paris Chemical), all dissolved in physiological saline; (+)bicuculline (Sigma Chemical Co.) suspended in Tween 80 and adjusted to the appropriate volume with physiological saline; diazepam (Valium, Roche Products) dissolved in a minimum amount of polyethylene glycol 400 (Fluka AG, Buchs) and adjusted to the appropriate volume with physiological saline. All drugs were administered intraperitoneally (i.p.) in a volume of 1 ml per 100 g body weight. Control animals received equal-volume injections of the vehicle.

The activity of the drugs used was not affected by the vehicle. Fresh drug solutions were prepared daily throughout the study. The pretreatment times prior to the injection of quinine were as follows: muscimol 1 h, AOAA 20 min, DABA 30 min, baclofen 30 min, bicuculline 10 min, diazepam 20 min, phenobarbitone 10 min and phenytoin 30 min. The pretreatment times as well as the doses used were established by preliminary studies in our laboratory.