sialic acid determinations[18,19]. Since assay of plasma LSA is relatively simple and has better specificity and sensitivity in patients and normal individuals studied, it could be useful as a prognostic determinant in a variety of neoplastic conditions.

Acknowledgments. This study was supported by NIH Grant # RR08198.

1 Emmelot, P., Eur. J. Cancer *9* (1973) 319.
2 Spiro, R. G., N. Engl. J. Med. *281* (1969) 991.
3 Spiro, R. G., N. Engl. J. Med. *281* (1969) 1043.
4 VanBeek, M. P., Smets, L. A., Emmelot, P., Cancer Res. *33* (1973) 2913.
5 Warren, L., Fuhrer, J. P., and Buck, C. A., Proc. natl Acad. Sci. USA *43* (1972) 1838.
6 Lipton, A., Harvey, H. A., and Delong, S., Cancer *43* (1979) 1766.
7 Silver, H.K.B., Karin, K. A., Archiblad, E. L., and Salinas, F. A., Cancer Res. *39* (1979) 5036.
8 Hogan-Ryan, A., Fennelly, J. J., Jones, J., Cantevell, B., and Duffy, M. J., Br. J. Cancer *41* (1980) 587.
9 Dwivedi, C., Dixit, M., Kumar, S. S., Reddy, H., Semenya, K. A., and Hardy, R. E., J. Med. *18* (1987) 323.
10 Dunzendorfer, U., Katopodis, N., Dnistrian, A. M., Stock, C. C., Schwartz, M. K., and Whitmore, W. F. Jr, Invest. Urol. (1981) 194.
11 Dnistrian, A. M., Schwartz, M. K., Katopodis, N., Fracchia, A. A., and Stock, C. C., Cancer *50* (1982) 1815.
12 Dnistrian, A. M., and Schwartz, M. K., Clin. Chem. *27* (1981) 1737.
13 Munjal, D. D., Picken, J., and Pritchard, J., Diagnos. Immun. *2* (1984) 36.
14 Shamberger, R. S., Anticancer. Res. *6* (1986) 717.
15 Horgan, I. E., Clinica chim. Acta *118* (1982) 327.
16 Ebril, K. M., Jones, J. D., and Klee, G. G., Cancer *55* (1985) 404.
17 Kakari, S., Michaelidis, G., Besbeas, S. T., Federigos, A. S., Tsitsoura, M., and Pulaki, E., Anticancer Res. *4* (suppl. 1) (1984) 3.
18 Dwivedi, C., Dixit, M., and Hardy, R. E., J. Med. *15* (1984) 379.
19 Dwivedi, C., Dixit, M., and Hardy, R. E. Cancer Detect. Preven. *11* (1988) 191.

0014-4754/90/010091-04$1.50 + 0.20/0

Deficiency of kallikrein-like enzyme activities in cerebral tissue of patients with Alzheimer's disease

T. Aoyagi, T. Wada, M. Nagai, F. Kojima, S. Harada, T. Takeuchi, H. Takahashi *, K. Hirokawa * and T. Tsumita *

*Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawaku, Tokyo 141 (Japan), and * Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashiku, Tokyo 173 (Japan)*
Received 6 March 1989; accepted 13 June 1989

Summary. We examined the changes in the intracerebral activities, at the time of postmortem autopsy, in patients with Alzheimer's disease. When compared with the control group, the activity of kallikrein-like enzyme was significantly decreased, while prolyl endopeptidase activity increased, in the patients group. Aprotinin inhibited 50% of the activity of the former enzyme at 2×10^{-7} M. Taken together with the results of a multivariate study, the above findings may indicate that intracerebral kallikrein deficiency plays an important role in the pathogenesis of Alzheimer's disease.
Key words. Alzheimer's disease; brain; proteases; kallikrein; prolyl endopeptidase.

Proteases not only govern protein metabolism in general, but are also concerned with turnover of the structural proteins of cells, and are thus closely related to cell functions. Because of this, many investigators have tried to connect age-related changes in cell functions with protease changes. Recently, several reports were published which suggested the accumulation of abnormal protein in the brains of patients with Alzheimer's disease[1-4]. Also, as more direct evidence, 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 during aging[5]. These reports prompted us to study the protease changes in the brain of patients with Alzheimer's disease.

Materials and methods

We selected 1 case of typical Alzheimer's disease (60-year-old female), 6 cases of senile dementia of the Alzheimer type (5 females, 84.8 ± 3.5 years; 1 male, 91 years) and 6 control subjects (3 males, 88.7 ± 5.7 years; 3 females, 72.3 ± 1.3 years). Histologically, brains from the 7 cases with dementia had senile plaques and neurofibrillary tangles in various amounts, but no vascular lesions detectable by routine examination. Control subjects had neither a clinical record of dementia nor pathologically significant lesions in the brain. The cerebral tissues had been stored at the time of autopsy in a deep freeze at −70 °C. In all cases the occipital lobe of the brain was used for enzymatic examination. The brain homogenates were prepared in phosphate-buffered saline (PBS, pH 7.2) by using a tissue homogenizer, Ultraturrax, at the maximum speed for 1 min. The homogenate was centrifuged (3000 g for 20 min), and the supernatant fluid was withdrawn for the measurement of enzymatic activities. The diagnosis was made according to pathological and clinical findings.

Determination of enzyme activities. The substrates, enzymes and their sources were as follows (see table 1 for abbreviations): Glu · NA, Arg · NA, Pro · NA, Gly-Arg · NA, Lys-Ala · NA, and Gly-Pro · NA from

Table 1. List of the proteases measured and their substrates.

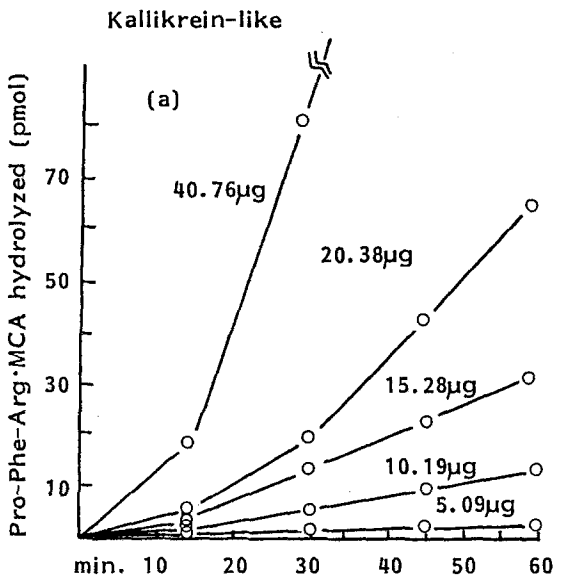
Enzyme	Abbreviation	Substrate	Reference for assay method
Aspartate aminopeptidase (EC3.4.11.7)	AP-A	Glu · NA	(6)
Arginine aminopeptidase (EC3.4.11.6)	AP-B	Arg · NA	(6)
Proline aminopeptidase (EC3.4.11.5)	Pro-IP	Pro · NA	(7)
Dipeptidyl aminopeptidase I (EC3.4.14.1)	DAP-I	Gly-Arg · NA	(8)
Dipeptidyl aminopeptidase II (EC3.4.14.2)	DAP-II	Lys-Ala · NA	(9)
Dipeptidyl aminopeptidase III (EC3.4.14.4)	DAP-III	Arg-Arg · NA	(10)
Dipeptidyl aminopeptidase IV (EC3.4.14.5)	DAP-IV	Gly-Pro · NA	(11)
Prolyl endopeptidase (EC3.4.21.26)	Post-Pro-Enz	Z-Gly-Pro · NA	(7)
Cathepsin B (EC3.4.22.1)	Cathepsin B	Z-Arg-Arg · NA	(12)
Tissue kallikrein (EC3.4.21.35)	Kallikrein	Pro-Phe-Arg · MCA	(13)
Trypsin (EC3.4.21.4)	Trypsin	Boc-Gln-Ala-Arg · MCA	(14)
Leukocyte elastase (EC3.4.21.37)	Elastase	Suc-Ala-Pro-Ala · MCA	(15)

Abbreviations used: Glu · NA, L-glutamic acid β-naphthylamide hydrochloride; Arg · NA, L-arginine β-naphthylamide hydrochloride; Pro · NA, L-proline β-naphthylamide hydrochloride; Gly-Arg · NA, glycyl-L-arginine β-naphthylamide; 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; Z-Gly-Pro · NA, carbobenzoxyglycyl-L-proline β-naphthylamide; Z-Arg-Arg · NA, carbobenzoxyarginyl-L-arginine β-naphtylamide; Pro-Phe-Arg · MCA, L-prolyl-L-phenylalanyl-L-arginine · 4-methylcoumaryl-7-amide; Boc-Gln-Ala-Arg · MCA, t-butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine · 4-methylcoumaryl-7-amide; suc-Ala-Pro-Ala · MCA, succinyl-L-alanyl-L-prolyl-L-alanine · 4-methylcoumaryl-7-amide.

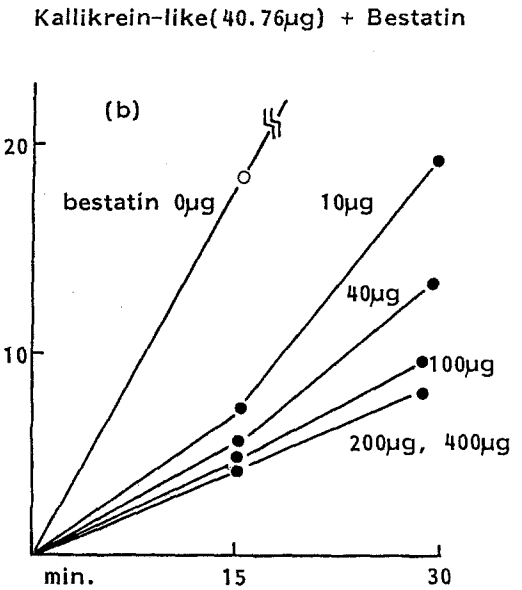
Bachem Feinchemikalien AG, Bubendorf, Switzerland; Pro-Phe-Arg · MCA, Boc-Gln-Ala-Arg · MCA, and Suc-Ala-Pro-Ala · MCA from Peptide Institute Inc, Osaka, Japan; soybean trypsin inhibitor (SBTI), aprotinin, human plasma kallikrein and porcine pancreas kallikrein from Sigma Chemical Company, St. Louis, USA. Z-Gly-Pro · NA and Z-Arg-Arg · NA were synthesized in our laboratory.

The supernatant fluids of the homogenates were dispensed into test tubes (1.5 × 10 cm) containing PBS for aminopeptidase assay, and 0.1 M Tris-HCl buffer (pH 8.0) for endopeptidases, to which the respective substrates were added, followed by incubation for 30 min at 37 °C. 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. For the assays, the units of the enzyme activities were expressed as nmols of reaction products generated during one minute of incubation per mg protein (nmol/min/mg protein).

Bestatin, inhibiting AP-B, Leu-AP, Ala-AP, tripeptidyl-AP, tetrapeptidyl-AP etc., and leupeptin, inhibiting plasmin, trypsin, kallikrein, papain, cathepsin B etc., have been found in culture filtrates of streptomyces strains [16].



a Time-course of kallikrein-like activity in mouse brain. The hydrolysis of Pro-Phe-Arg · MCA was examined in the supernatant of brain homogenate by the method of Nagase et al.[14]. Enzyme activities are expressed as pmol of 4-methylcoumaryl-7-amide (MCA) per the amount of the enzyme added (40.76 μg, 20.38 μg, 15.28 μg, 10.19 μg, 5.09 μg, o——o). Kallikrein-like activity is expressed as protein content in supernatant of brain homogenate.



b Effect of bestatin on kallikrein-like activity in mouse brain. Kallikrein-like activities (40.76 μg) are expressed as pmol of MCA per the amount of bestatin added (10 μg, 40 μg, 100 μg, 200 μg, 400 μg, ●——●).

SBTI is an inhibitor of plasma kallikrein. Aprotinin is an inhibitor of kallikrein, trypsin, chymotrypsin etc. Protein was determined by the method of Lowry et al. [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 performed preliminary studies.

Figure (a) illustrates the time course of the kallikrein-like activity in the homogenate of the murine brain. Nonlinearity of the catalytic activity of this enzyme increased with the amount of the enzyme added. Figure (b) showed, however, that the addition of bestatin at a dose of 200 to 400 μg/ml completely straightened the reaction curves.

Statistical analysis. Multiple discriminant analysis [18] was adopted to find a linear composite (i.e., the weighted sum) of all the measured variables (i.e., enzymatic activities) that had the property of maximally separating the two groups, the Alzheimer's disease patients and the control.

Results

Table 2 compares the intracerebral activities of 12 kinds of proteases between the two groups, control and pathologic. The activity of kallikrein-like enzyme was significantly decreased, while that of prolyl endopeptidase (Post-Pro-Enz) was increased in the patient group when compared with the control group. Besides the kallikrein-like activity, we found weak cleavage of the substrates specific to various endopeptidases including elastase, trypsin and chymotrypsin. Since it is unlikely that these particular enzymes occur in brain tissue in active forms, it seems that some endopeptidases of lysosomal origin were responsible for these endopeptidase activities.

In order to examine the properties of the kallikrein-like activity in the brain, we tested the inhibition patterns of several inhibitors. The kallikrein-like enzyme activity was inhibited both by leupeptin and aprotinin, without any significant difference in reactivity between control and diseased brain. The 50% inhibition concentration (IC_{50}) of leupeptin was 3×10^{-9} M, whereas that of aprotinin was 2×10^{-7} M. SBTI showed no inhibition within the same range of concentration, excluding the possibility that the kallikrein-like activity came from the plasma kallikrein in cerebral blood vessels.

Table 2. Changes in protease activities in brain of patients with Alzheimer's disease.

Enzyme	Specific activity (Mean ± SD, nmol/min/mg protein) Normal (n = 6)	Alzheimer (n = 7)
AP-A	0.93 ± 0.26	1.02 ± 0.39
AP-B	17.65 ± 10.52	19.97 ± 8.71
Pro-IP	10.61 ± 4.20	12.99 ± 4.33
DAP-I	0.35 ± 0.16	0.22 ± 0.13
DAP-II	7.09 ± 2.55	5.47 ± 2.40
DAP-III	7.65 ± 1.07	7.95 ± 2.02
DAP-IV	0.48 ± 0.22	0.61 ± 0.25
Post-Pro-Enz	2.31 ± 1.52	4.68 ± 1.39*
Cathepsin B	21.38 ± 7.72	14.21 ± 5.37
Kallikrein	0.037 ± 0.012	0.019 ± 0.003**
Trypsin	0.061 ± 0.061	0.023 ± 0.011
Elastase	0.132 ± 0.117	0.26 ± 0.11

* $p < 0.05$; ** $p < 0.01$; Normal: 80.5 ± 11.0 (Mean ± SD) years old; Alzheimer: 80.6 ± 12.0 (Mean ± SD) years old.

Table 3. Correlation of various enzymatic activities with the discriminant function score.

Enzyme	Correlation	Enzyme	Correlation
AP-A	0.18	Post-Pro-Enz	0.63*
AP-B	0.20	Cathepsin B	−0.54
Pro-IP	0.35	Kallikrein	−0.79**
DAP-I	−0.44	Trypsin	−0.46
DAP-II	−0.32	Elastase	0.45
DAP-III	0.14		
DAP-IV	0.17	(Postmortem hours	0.06)

* $p < 0.05$; ** $p < 0.01$

To elucidate the meaning of the multiple enzymatic changes in the disease, we performed a multivariate study using discriminant function analysis. The principle was to find a linear composite (i.e., a weighted sum) of all the enzymatic activities measured that had the property of maximally separating the patients with Alzheimer's disease from control subjects. With the use of the discriminant function score, the two groups of subjects were completely separated with no overlapping. The correlation of each variable (i.e., each enzymatic activity) to the score is called the structure coefficient, and is presented in table 3. As can be seen, the highest correlation, a negative correlation of −0.78, was seen with the activity of kallikrein-like enzyme. The activity of Post-Pro-Enz showed the second highest, in this case positive, correlation of 0.63. In addition, we calculated the correlation between the discriminant function score and the length of the time which lapsed from death to the postmortem autopsy. This correlation was only 0.06.

Discussion

In the present study, we utilized various inhibitors, including those discovered in our laboratory [16], for enzyme assays and for the identification of the enzymatic activities in the brain.

The synthetic substrates used in the present study for endopeptidase assays are known to have a high specificity for each target enzyme. However, since organ homogenates contain numerous unknown enzymes which may act on the substrates in the reaction process of the assay, we utilized bestatin to suppress extra enzyme reactions. This maneuver did indeed enable us to secure the linearity of the endopeptidase assays.

Of the 12 enzymes which were examined, the activities of only two, kallikrein-like enzyme and propyl endopeptidase (Post-Pro-Enz), showed statistically significant differences between the two groups of subjects. In particular, the activity of kallikrein-like enzyme showed the highest correlation to the score of discriminant function,

which is to separate maximally the two groups of subjects.

Interestingly, the above two enzymes have been suggested to have some relationships to senile dementia. Kallikrein is the term used for those serine proteinases which liberate kinins from kininogen by limited proteolysis. Activity of this type of protease has been found to exist not only in plasma but also in saliva, submaxillary gland, urine, and various organs[19,20]. In addition to the inhibition by leupeptin, aprotinin, which is known to be a specific inhibitor of kallikrein, inhibited the kallikrein-like activity at the concentration lower than 10^{-6} M. Very recently, our preliminary study using a monoclonal antibody against kallikrein showed the existence of this proteinase in brain tissue. These observations indicate that the kallikrein-like activity we found does indeed represent kallikrein itself.

Conventionally, kallikrein has been related to peripheral blood circulation because of its action on kininogen to generate bradykinin. Hence it has been used as a medicine to treat peripheral circulatory disturbance including cerebral arteriosclerosis[21].

On the other hand, Post-Pro-Enz is known to play an important role in the degradation of biologically active peptides such as vasopressin, which may facilitate the process of learning and memory[22,23]. Moreover, several inhibitors of this enzyme have been reported to have anti-amnesic effects in mice[24]. Accordingly, in spite of the lack of direct evidence, it seems to be reasonable to combine these findings concerning the roles of prolyl endopeptidase (Post-Pro-Enz) in senile dementia. The present results may give additional support for such a notion. It may be possible to link the above two enzymatic changes together from the standpoint of kinin metabolism, since kallikrein increases the generation, whereas Post-Pro-Enz increases the degradation of bradykinin.

Finally, the protease changes found in the present study, all together, may be related to the accumulation of abnormal proteins such as lipofuscin, paired helical filaments (PHF), tau-protein, 68K protein, ubiquitin, and amyloid beta protein precursor in the brain in Alzheimer's disease, suggested by many investigators recently[1-5,25,26]. Thus it is tempting to speculate that the deficiency of kallikrein, either by causing a disturbance in intracerebral blood circulation or by some more direct mechanism, plays a central role as a trigger leading to the abnormal metabolism of protein.

Acknowledgments. This work was partly supported by a grant-in-aid for New Drug Development Research from the Ministry of Health and Welfare, Japan.

1 Ihara, Y., Nukina, N., Miura, R., and Ogawara, M., J. Biochem. *99* (1986) 1807.
2 Selkoe, D. J., Bell, D. S., Podlisny, M. B., Price, D. L., and Cork, L. C., Science *235* (1987) 873.
3 Perry, G., Friedman, R., Shaw, G., and Chau, V., Proc. natl Acad. Sci. USA *84* (1987) 3033.
4 Mori, H., Kondo, J., and Ihara, Y., Science *235* (1987) 1641.
5 Ivy, G. O., Schottler, F., Wenzel, J., Baudry, M., and Lynch, G., Science *226* (1984) 985.
6 Aoyagi, T., Wada, T., Kojima, F., Nagai, M., and Umezawa, H., J. clin. Invest. *67* (1981) 51.
7 Aoyagi, T., Wada, T., Kojima, F., Nagai, M., Okubo, M., Masaki, Y., and Umezawa, H., J. Appl. Biochem. *7* (1985) 273.
8 McDonald, J. K., and Schwabe, C., in: Proteinases in Mammalian Cells and Tissues, p. 314. Ed. A. J. Barrett. Elsevier/North-Holland Biomedical Press, Amsterdam 1977.
9 McDonald, J. K., Leibach, F. H., Grindeland, R. E., and Ellis, S., J. biol. Chem. *243* (1968) 4143.
10 Ellis, S., and Nuenke, J. M., J. biol. Chem. *242* (1967) 4623.
11 Aoyagi, T., Wada, T., Kojima, F., Nagai, M., Osanai, M., Imahori, K., and Umezawa, H., Biotech. appl. Biochem. *8* (1986) 23.
12 Barrett, A. J., and Kirschke, H., in: Methods in Enzymology, p. 535. Ed. L. Lorand. Academic Press, New York 1981.
13 Nagase, H., and Barrett, A., Biochem. J. *193* (1981) 187.
14 Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T., and Sakakibara, S., Eur. J. Biochem. *172* (1988) 17.
15 Mumford, R. A., Strauss, A. W., Powers, J. C., Pierzchala, P. A., Nishino, N., and Zimmerman, M., J. biol. Chem. *255* (1980) 2227.
16 Aoyagi, T., in: Horizons on Antibiotic Research, p. 149. Eds B. D. Davis, T. Ichikawa, K. Maeda and L. A. Mitscher. Japan Antibiotic Research Association, Tokyo 1988.
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
18 Kendall, M., in: Multivariate Analysis, p. 13. Griffin, London 1975.
19 Fujimoto, Y., Moriya, H., and Moriwaki, C., J. Biochem. *74* (1973) 247.
20 Matsuda, Y., Miyazaki, K., Moriya, H., Fujimoto, Y., Hojima, Y., and Moriwaki, C., J. Biochem. *80* (1976) 671.
21 Windholz, M., Budavari, S., Blumetti, S., and Otterbein, E. S., in: The Merck Index, 10th edn, p. 5112. Merck & Co., Rahway, N.J. 1983.
22 Wied, D., Gaffori, O., Ree, J. M., and Jong, W., Nature *308* (1984) 276.
23 Brinton, R. E., Gehlert, D. R., Wamsley, J. K., Wan, Y. P., and Yamamura, H. I., Life Sci. *38* (1986) 443.
24 Kubota, A., Nakajima, T., Okada, T., Hayashi, T., and Nakamura, K., Jap. J. Pharm. *33* (1983) suppl. 67.
25 Kidd, M., Nature *197* (1963) 192.
26 Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M. Masters, C. L., Grzeschik, K. H., Malthaup, G., Beyreuther, K., and Muller, H. B., Nature *325* (1987) 733.

0014-4754/90/010094-04$1.50 + 0.20/0