研究成果の刊行に関する一覧表

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tomoyuki Kida, Kouzin Kamino, Mitsuko Yamamoto, Daisuke Kanayama, Toshihisa Tanaka, Takashi Kudo , Masatoshi Takeda	C677T polymorphism of methylenetetrahydrofolkate reductase gene affects plasma homocysteine level and is gwenetic fctor for late-onset Alzheimer's disease	Psychogeraitrics	4	4-10	2004
Okochi M, Fukumori A, Satoh Y, Aidaralieva, N, Tanii H, Kamino K, Tanaka T, Kudo T, Takeda M.	Alzheimer' s-secretase mechanism produces Amyloid- β -protein like peptides simultaneously with release of intracellular signaling fragments	Molecular Neurobiology of Alzheimer Disease and Related Disorders	Karger Press	31-41	2004
Aremu DA, Meshitsuka S.	Accumulation of aluminum by primary cultured astrocytes from aluminum amino acid complex and its apoptotic effect.	Brain Research	1031	284-296	2005
Aremu DA, Sakurai A, Meshitsuka S.	Uptake of aluminum amino acid complexes in cultured astrocytes.	Biomedical Research Trace Elements	15(1)	66-68	2004
Numakawa T, Yagasaki Y, Ishimoto T, Okada T, Suzuki T, Iwata N, Ozaki N, Taguchi T, Tatsumi M, Kamijima K, Straub RE, Weinberger DR, Kunugi H, Hashimoto R.	Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia.	Hum Mol Genet	13(21)	2699-2708	2004
Shigetsugu H, Matsumoto M, Kamura T, Murayama M, Chui D-H, Planel E, Takahashi R, Nakayama K-I, and Takashima A	U-box protein carboxyl terminus of Hsc7-interacting protein (CHIP) mediates poly-ubiquitylation preferentially on four-repeat Tau and is involved in neurodegeneration of tauopathy	J. Neurochem	91	299-307	2004
Atsuko Nishikawa, Takayuki Manabe, Taiichi Katayama, Takashi Kudo, Shinsuke Matsuzaki, Takeshi Yanagita, Hiroaki Okuda, Yoshio Ban, Masaya Tohyama	Novel function of PS2V: change in conformation of tau proteins.	*Biochem Biophys Res Commun*	28;318(2)	435-8	2004

ORIGINAL ARTICLE

C677T polymorphism of methylenetetrahydrofolate reductase gene affects plasma homocysteine level and is a genetic factor of late-onset Alzheimer's disease

Tomoyuki KIDA, Kouzin KAMINO, Mitsuko YAMAMOTO, Daisuke KANAYAMA, Toshihisa TANAKA, Takashi KUDO and Masatoshi TAKEDA

Division of Psychiatry and Behavioral Proteomics, Department of Post Genomics and Diseases, Osaka University Graduate School of Medicine, Osaka, Japan

Correspondence: Dr Kouzin Kamino MD, PhD, 2-2, D3, Yamadaoka, Suita, Osaka 565–0871, Japan. Email: kkamino@psy.med.osaka-u.ac.jp

Received 25 November 2003; accepted 14 January 2004.

Abstract

Background: Elevated plasma homocysteine levels are known as a risk for atherosclerotic vascular disease and venous thrombosis and have been shown as a risk for late-onset Alzheimer's disease (LOAD).

Method: To examine the effect of genetic factors predisposing to elevated plasma homocysteine levels on the occurrence of LOAD, we determined the genotype of a C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene and a variable number tandem repeat (VNTR) spanning exon 13–intron 13 boundary of cystathionine β-synthase (CBS) gene in patients with LOAD and community-based control subjects.

Results: Logistic regression indicated that the MTHFR-T allele was a risk for LOAD ($P < 0.05$), independently from apolipoprotein E-ε4 (APOE-ε4) allele. Kaplan–Meier tests showed that in APOE-ε4 non-carriers, individuals with the MTHFR-TT genotype have occurences of LOAD earlier than those with the MTHFR-CC genotype ($P < 0.05$). Multiple regression analysis indicates that MTHFR-T allele increases plasma homocysteine levels ($P = 0.0002$), while the number of X chromosomes decreases ($P = 0.01$). Plasma homocysteine level was not correlated with age, plasma albumin reflecting nutritional condition, and the dose of APOE-ε4 allele. The CBS-20 VNTR allele showed the same trend to increase plasma homocysteine level as the MTHFR-T allele, but a risk effect for LOAD was not evident.

Conclusion: A genetic propensity for elevated plasma homocysteine levels, explained by the MTHFR-T allele encoding defective enzymatic function, is involved in the development of LOAD, particularly in APOE-ε4 non-carriers, and that homocysteine metabolism could be a preventive target to LOAD in the elderly.

Key words: Alzheimer's disease, apolipoprotein, cystathionine β-synthase, genetic, homocysteine, methylenetetrahydrofolate reductase, risk.

INTRODUCTION

The major cause of dementia in the elderly is late-onset Alzheimer's disease (LOAD) both among Caucasians and, after 1990s, the Japanese elderly.[1] This trend could be caused by preventive intervention and advanced treatment of cerebrovascular stroke (such as antihypertensive treatment), dietary salt restriction and protein supplement, improved indoor air-conditioning,[2] and global warming. A cohort study in Caucasians showed that elevated plasma homocysteine level is a risk factor for cognitive decline in the elderly, notably for LOAD.[3,4] However, elevated plasma homocysteine level is a risk factor not only for atherosclerotic vascular disease and venous thrombosis but also cerebrovascular disease.[5–9] Plasma homocysteine level is modified by dietary environment; for example, loading of methionine induced elevated plasma homocysteine level under low vitamin B12 supplement.[10] Although, it was noted that both fasting and postmethionine-load

hyperhomocysteinemia is inherited in many of the instances.[11] C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene is one of genetic factors affecting plasma homocysteine levels.[12] Demented patients with multiple infarcts had a higher frequency of the MTHFR-TT genotype than those without.[8] However, it remains undetermined whether the MTHFR-T allele modifies the risk for either vascular dementia or LOAD.[13–16] Cystathionine β-synthase (CBS) gene is another genetic factor to determine plasma homocysteine levels, since a 31-bp variable number tandem repeat (VNTR) spanning the exon 13–intron 13 boundary of the CBS gene is related to plasma homocysteine levels.[17] Subjects with Down syndrome show the appearance of senile plaque in the brain in their thirties, a pathological hallmark of Alzheimer's disease (AD). CBS gene is localized in the critical region of Down syndrome, at 21q22.3,[18] but the genetic association between LOAD and the CBS gene, to our knowledge, has not been reported. To elucidate how genetic factors related to plasma homocysteine level modify the occurrence of LOAD, we performed a case–control study to examine the relation of plasma homocysteine level and genetic polymorphisms modifying plasma homocysteine in patients with LOAD, and evaluated how these genetic factors contribute to the occurrence of LOAD.

SUBJECTS AND METHODS

Subjects

Patients with AD were diagnosed as having probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA).[19] Elderly subjects, living healthily and independently at home, were recruited in Suita City, Osaka, Japan. No cognitive impairment in these subjects was found by the questionnaire including date, orientation, past history and medical records. After written informed consent to participate in the present study was obtained, peripheral blood was drawn from the patients and these population-based control subjects. The Genome Ethical Committee of Osaka University Graduate School approved this procedure. The age of the patients (n = 196) at blood withdrawal was 79.2 years + 7.0 (mean + SD), range 65–98 years, and that of age-matched control subjects (n = 385) was 75.4 years + 5.0, range 65–92 years. Age at onset of AD was 74.7 years + 6.9, range 65–94 years. Peripheral blood was drawn into ethylenediaminetetraacetic acid dinatrium (EDTA2Na), and plasma and cell fractions were separated within 6 h. DNA was extracted from the cell fraction using a QIAamp DNA blood kit (Qiagen, Hilden, Germany) and stored at 4°C. Plasma was stored at 80°C until use.

Genotyping

The genotype of the APOE gene was determined using polymase chain reaction–restriction fragment length polymorphism (PCR–RFLP) according to the procedure by Wenham *et al.*[20] The C677T polymorphism of the MTHFR gene was genotyped by a PCR–RFLP method using the *Hin*fI restriction enzyme according to the procedure described previously.[21]

The 31 bp VNTR region in the CBS gene was amplified using primers CBS3: 5′-GGAATGGT GACGCTTGGGAACAT-3′ and CBS4: 5′-ACTTGTAAA GTGGGTGCTTCTCAGC-3′, and PCR product were electrophoresed in 2.5% agarose gel containing ethidium bromide and visualized on an ultraviolet transilluminator. The 31 bp tandem-repeat polymorphic alleles were determined by the comparison with the most frequently observed 796 bp alleles harboring 18 repeats.[17,22]

Plasma homocysteines and albumin

For the patients with LOAD (n = 77) we examined the relationship between the allelic doses of the MTHFR-T allele and CBS-repeat doses with plasma total homocysteine level. The concentration of plasma homocysteine was measured by the high-pressure liquid chromatography (HPLC), that included derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (ABD-F) and postcolumn fluorescence detection.[23] To test the effect of nutrition, plasma albumin level was also measured by the BCG method described previously.[24]

Statistics

The allele and genotype frequencies was compared by χ^2-tests, and P-value <0.05 was considered significant after correction by the number of comparisons. Multiple logistic regression of the occurrence of LOAD with the dose of the APOE-ε4, MTHFR-T, and CBS-20 VNTR alleles were examined. Kaplan–Meier survival curves free from LOAD were examined by the Mantel-Cox logrank test. The correlation of plasma

homocysteine level with age, dose of X chromosome, plasma albumin level, dose of the APOE-ε4, MTHFR-T, and CBS-20 VNTR alleles was examined by multiple regression. *P*-values <0.05 was considered significant.

RESULTS

Allele and genotype frequencies of methylenetetrahydrofolate reductase-C766T and cystathionine β-synthase-variable number tandem repeat polymorphisms

We examined the genotype and allele frequencies of the MTHFR-C766T and CBS-VNTR polymorphisms in patients with LOAD and population-based non-demented controls (Tables 1 and 2). Genotype distributions of both of the polymorphisms were in Hardy–Weinberg equilibrium. The genotype frequency of the MTHFR-C766T polymorphism was not significantly different between the groups, but the allele frequency of MTHFR-T allele in the patient group was relatively higher than that in the control group (Table 1). When the subjects were divided into APOE-ε4 carrier and non-carrier, the MTHFR-T allele in the patient group was significantly more frequent than that in the control group in APOE-ε4 non-carriers ($P < 0.02$). The same trend was also found in APOE-ε4 carrier, but not significantly. However, we did not find any significant difference in the CBS-VNTR allele and genotype frequencies between the groups (Table 2).

To incorporate genetic interactions in risk effects, we performed a logistic regression of the APOE-ε4, MTHFR-T and CBS-20 VNTR alleles for the occurrence of LOAD (Table 3). We detected a significant risk effect of the APOE-ε4 (odds ratio = 5.2, 95% CI = 3.51–7.59, $P < 0.0001$) and also a marginally significant effect of the MTHFR-T allele (odds ratio = 1.4, 95% CI = 1.02–1.85, $P = 0.04$), but the CBS-20 VNTR allele did not show any significant effect. The risk effect of the MTHFR-T allele was more prominent

Table 1 Genotype and allele frequencies of methylenetetrahydrofolate reductase C766T polymorphism in patients with late-onset Alzheimer's disease (LOAD) and controls

	Genotype (frequency)			Allele (frequency)	
Group	CC	CT	TT	C	T
All subjects					
Patients (n = 194)	64 (0.330)	98 (0.505)	32 (0.164)	226 (0.582)	162 (0.418)
Controls (n = 379)	144 (0.380)	193 (0.509)	42 (0.111)	481 (0.635)	277 (0.365)
APOE-ε4(+)					
Patients (n = 102)	38 (0.373)	52 (.510)	12 (0.118)	128 (0.627)	76 (0.373)
Controls (n = 60)	25 (0.417)	30 (0.500)	5 (0.083)	80 (0.667)	40 (0.333)
APOE-ε4(–)					
Patients (n = 92)	26 (0.282)	46 (0.500)	20 (0.217)	98 (0.533)	86 (0.467)*
Controls (n = 319)	119 (0.373)	163 (0.511)	37 (0.116)	401 (0.629)	237 (0.37)

The genotypes of the methylenetetrahydrofolate reductase (MTHFR) polymorphism of two patients and six controls were not determined because of the insufficient polymase chain reaction amplification. Allele frequency of the MTHFR polymorphism in APOE-ε4 (–) group was significantly different between the patients and controls ($P = 0.02$).

Table 2 Genotype frequencies of the cystathionine β-synthase (CBS) polymorphism in patients with late-onset Alzheimer's disease (LOAD) and controls

	Genotype (frequency)				Allele (frequency)		
Group	18/18	18/20	17/18	20/20	17	18	20
All subjects							
Patients (n = 190)	144 (0.758)	45 (0.237)	0 (0.000)	1 (0.005)	0 (0.000)	333 (0.876)	47 (0.124)
Controls (n = 384)	285 (0.742)	91 (0.237)	2 (0.005)	6 (0.016)	2 (0.003)	663 (0.863)	103 (0.134)
APOE-ε4(+)							
Patients (n = 98)	70 (0.714)	27 (0.276)	0 (0.000)	1 (0.010)	0 (0.000)	167 (0.852)	29 (0.148)
Controls (n = 60)	49 (0.817)	9 (0.150)	0 (0.000)	2 (0.033)	0 (0.000)	107 (0.892)	13 (0.108)
APOE-ε4(–)							
Patients (n = 92)	74 (0.804)	18 (0.196)	0 (0.000)	0 (0.000)	0 (0.000)	166 (0.902)	18 (0.098)
Controls (n = 324)	236 (0.728)	82 (0.253)	2 (0.006)	4 (0.012)	2 (0.003)	556 (0.858)	90 (0.139)

The genotypes of the CBS polymorphism of six patients and one controls were not determined because of the insufficient PCR amplification.

among APOE-ε4 non-carriers (odds ratio = 1.5, 95% CI = 1.04–2.14, $P = 0.03$).

Methylenetetrahydrofolate reductase-TT genotype and onset of late-onset Alzheimer's disease

To examine the genetic effect of the defective MTHFR genotype, the common MTHFR-CC genotype and the defective MTHFR-TT genotype was compared for the survival free from LOAD. In all subjects, the subjects with MTHFR-TT genotype showed a trend to develop LOAD faster than those with MTHFR-CC genotype (Fig. 1a). This trend was observed in both APOE-ε4 non-carriers (Fig. 1b) and APOE-ε4 carriers (Fig. 1c). However, the significant difference was only supported in APOE-ε4 non-carriers ($P < 0.05$) (Fig. 1b). We did not find any difference of the survival rate free from LOAD between subjects with the CBS-20/20 VNTR genotype and those with the CBS-18/18 VNTR genotype (data not shown).

Table 3 Logistic regression of doses of the APOE-ε4, methylenetetrahydrofolate reductase (MTHFR)-T and cystathionine β-synthase (CSB)-20 variable number tandem repeat (VNTR) alleles for the occurrence of late-onset Alzheimer's disease (LOAD)

Allele	Odds ratio (*P*-value)	95% Confidence interval
All subjects		
APOE-ε4	5.2*	3.51–7.59
MTHFR-T	1.4**	1.02–1.85
CBS-20 VNTR	0.8	0.55–1.27
APOE-ε4(+) subjects		
APOE-ε4	3.8	0.81–17.9
MTHFR-T	1.1	0.68–1.93
CBS-20 VNTR	1.2	0.61–2.51
APOE-ε4(–) subjects		
MTHFR-T	1.5***	1.04–2.14
CBS-20 VNTR	0.7	0.37–1.16

*$P < 0.0001$, **$P = 0.04$, ***$P = 0.03$.

Correlation of plasma homocysteine level with the methylenetetrahydrofolate reductase-T allele

To examine the effect of age, sex, nutrition, and risk alleles for the occurrence of LOAD on plasma homocysteine level, we performed a multiple regression of these parameters in patients with LOAD (Table 4). Among those, significant correlations with plasma homocysteine level were found in the dose of X chromosome (standerdized coefficient = – 0.266, $P = 0.01$) and that of the MTHFR-T allele (standaerdized coefficient = 0.404, $P = 0.0002$). The dose of the CBS-20 VNTR allele showed a trend to increase the plasma homocysteine level, but marginally not significant (standerdized coefficient = 0.185, $P = 0.08$). No significant effects were found with age, plasma albumin level, and the APOE-ε4 allele.

DISCUSSION

We confirmed that plasma homocysteine level in patients with LOAD is genetically affected by the C766T polymorphism of the MTHFR gene, and that the VNTR length of the CBS gene showed a similar trend to the MTHFR-T allele. The MTHFR-T allele results in an Ala222Val substitution, leading to reduced enzyme activity and increased thermolability, and relates to increased plasma homocysteine level.[21] The increase in the number of a 31-bp VNTR unit of the CBS gene shows a reduced activity of CBS caused by alternative splicing of exon 13, and relates with elevated plasma homocysteine level after

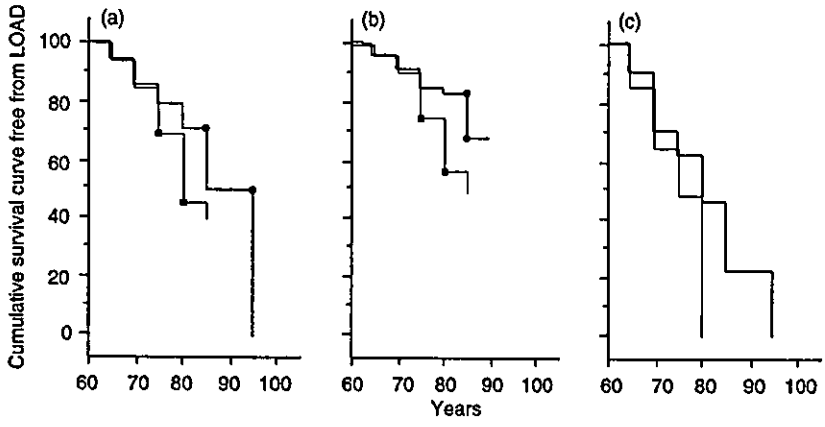

Figure 1 Kaplan–Meier survival curves free from late-onset Alzheimer's disease (LOAD). (●) subjects with the methylenetetrahydrofolate reductase (MTHFR)-CC genotype and (■) those with the MTHFR-TT genotype. Age of subjects is represented by seven groups: 65 years (ranged 65–67 years), 70 (ranged 68–72), 75 (ranged 73–77), 80 (ranged 78–82), 85 (ranged 83–87), 90 (ranged 88–92), and 95 (ranged 93–97). The curves were drawn for (a) all subjects; (b) APOE-ε4 carriers; and (c) APOE-ε4 non-carriers. Logrank test indicated a significant difference of the survival rate free from LOAD between the subjects in APOE-ε4 non-carriers ($P < 0.05$).

Table 4 Multiple regression of plasma homocysteine level with doses of the methylenetetrahydrofolate reductase (MTHFR)-T and cystathionine β-synthase (CBS)-20 variable number tandem repeat (VNTR) alleles

Factor	Coefficient (Standardized)	*t*-value
Age	0.095 (0.089)	0.785
X chromosome	−4.082 (−0.266)	−2.560*
Albumin	0.701 (0.038)	0.352
APOE-ε4	−0.136 (−0.012)	−0.108
MTHFR-T	3.816 (0.404)	3.925**
CBS-20 VNTR	2.574 (0.185)	1.754

*$P = 0.01$, **$P = 0.0002$.

methionine loading.[17] A gene–gene interaction between the MTHFR and CBS gene has been shown in plasma homocysteine level.[25]

We found a risk effect and a promotive effect on the occurrence of LOAD by the MTHFR-T allele in APOE-ε4 non-carriers. The same trend was also found in APOE-ε4 carriers, though not significantly. Several reports examined the risk effect of the MTHFR-T allele on the occurrence of LOAD, none of which found any significant effects.[13–16] We showed that the VNTR of the CBS gene did not associate with the occurrence of LOAD. Thus, elevated plasma homocysteine level in LOAD in itself merely reflects one of a vascular factor of LOAD, as shown in cerebrovascular dementia with multiple infarcts.[3,9]

It was well demonstrated that any positive association in LOAD other than APOE-ε4 allele are difficult to replicate, which likely caused by its weak effect. However, selection bias of patients as well as controls often affects the association studies. The frequency of the MTHFR-T allele in Japanese controls was similar to that reported by Wakutani *et al.*[16] We did not include either patients with cerebrovascular changes screened by neuroimaging studies. Therefore, it is unlikely that the patient groups in our study contained vascular dementia that was shown to associate with the MTHFR-T allele.[9] On the other hand, the risk effect of the MTHFR-T allele for coronary heart disease is well demonstrated,[26] and the link between LOAD and coronary heart disease has been noted in relation to cerebral amyloid deposition.[27] Thus, the risk effect for the other diseases might affect the notification of patients with LOAD. Although we found the relation between LOAD and the MTHFR-T allele, the risk effect of the MTHFR-T allele should be carefully confirmed by not only meta-analysis of a large number of case-control studies but prospective cohort studies for aging-related diseases. These studies will elucidate environmental differences to explain the different results of the association studies. Several studies indicated that plasma homocysteine level is related with cognitive function in the elderly,[28,29] though this relation remains controversial.[30,31] Neuroimaging studies evidenced that elevated plasma homocysteine level is related with cortical and hippocampal atrophy in non-demented subjects,[32,33] and with white matter changes in AD cases.[34] It was shown that hyperhomocysteinemia is related to the progression and increasing severity of LOAD.[35] On the other hand, homocysteine enhances the toxicity of beta amyloid in vascular smooth muscle cells, showing the relation of homocysteine with cerebral amyloid angiopathy.[36] One of the unifying mechanisms of this evidence is that elevated plasma homocysteine level could underlie the alteration of microcirculation in the brain, resulting in critically attained threshold of cerebral hypoperfusion.[37]

The CBS gene is localized at 21q22.3, inside the critical region of Down syndrome.[38,39] The brains with Down syndrome show the appearance of senile plaques, a pathological hallmark of LOAD, in their thirties,[40] and the activity of CBS is increased in Down syndrome caused by gene-dose effect, which is opposite to the reduction of the CBS activity in LOAD.[18] However, the content of hydrogen sulfides, endogenously produced from cysteine by CBS, was decreased in the brains with AD, and this decrease was associated with the reduction of S-adenosylmethionine (SAM), a CBS activator, catalyzed from methionine and ATP by methionine adenosyltransferase.[41] It was also reported that AA genotype of A2756G polymorphism of 5-methyltetrahydrofolate-homocysteine S-methyltransferase gene (MTR), also called as methionine synthase and encoded at 1q43, that catalyzes homocysteine to methionine, is an APOE-ε4 allele-independent risk factor for AD.[42] Since we also found that the MTHFR-T allele is an APOE-ε4 allele-independent risk factor for LOAD, folate cycle in homocysteine metabolism is likely to be involved in the development of LOAD. However, it remains undetermined how this metabolic regulation modifies the risk for LOAD.

In conclusion, we showed that the MTHFR-C766T polymorphism, a genetic factor correlated to elevated plasma homocysteine level, is related to the occur-

rence of LOAD. It was supported that control of plasma homocysteine could be beneficial to prevent the elderly from LOAD.

ACKNOWLEDGMENTS

We wish to thank the elderly people who participated in the present study. This study was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine and was supported by the Research for the Future Program, Japan Society for the Promotion of Science (JSPS).

REFERENCES

1 Shibayama H, Kobayashi H, Nakagawa M *et al.* Prevalence of dementia among the elderly in a Japanese community population – comparative study on the 1983 and 1996 survey. *Alchi Study Psychoger* 2001; **1**: 317–325.
2 Aylin P, Morris S, Wakefield J, Grossinho A, Jarup L, Elliott P. Temperature, housing, deprivation and their relationship to excess winter mortality in Great Britain 1986–96. *Int J Epidemiol* 2001; **30**: 1100–1108.
3 Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. *Arch Neurol* 1998; **55**: 1449–1455.
4 Seshadri S, Beiser A, Selhub J *et al.* Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med* 2002; **346**: 476–483.
5 Selhub J, Jacques PF, Bostom AG *et al.* Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *N Engl J Med* 1995; **332**: 286–291.
6 den Heijer M, Koster T, Blom HJ *et al.* Hyperhomocystinemia as a risk factor for deep-vein thrombosis. *N Engl J Med* 1996; **334**: 759–762.
7 Boushey CJ, Beresford SSA, Omenn GS, Motulsky AG. A meta-analysis of plasma homocysteine as a risk factor for arteriosclerosis vascular disease and the potential preventive role of folic acid. In: *International Congress on Homocysteine Metabolism: from Basic Science to Clinical Medicine*. Norwall: Kluwer Academic, 1995.
8 Clarke R, Lewington S, Donald A *et al.* Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002; **288**: 2015–2022.
9 Yoo JH, Choi GD, Kang SS. Pathogenicity of thermolabile methylenetetrahydrofolate reductase for vascular dementia. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1921–1925.
10 Mann NJ, Li D, Sinclair AJ *et al.* The effect of diet on plasma homocysteine concentrations in healthy male subjects. *Eur J Clin Nutr* 1999; **53**: 895–899.
11 Franken DG, Boers GHJ, Blom HJ, Cruysberg JRM, Trijbels FJM, Hamel BCJ. Prevalence of familial mild hyperhomocysteinemia. *Atherosclerosis* 1996; **125**: 71–80.
12 Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG. MTHFR 677C->T polymorphism and risk of coronary heart disease: a meta-analysis. *JAMA* 2002; **288**: 2023–2031.
13 Pollak RD, Pollak A, Idelson M, Bejarano-Achache I, Doron D, Blumenfeld A. The C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene and vascular dementia. *J Am Geriatr Soc* 2000; **48**: 664–668.
14 Bottiglieri T, Parnetti L, Arning E *et al.* Plasma total homocysteine levels and the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene: a study in an Italian population with dementia. *Mech Ageing Dev* 2001; **122**: 2013–2023.
15 Brunelli T, Bagnoli S, Giusti B *et al.* The C677T methylenetetrahydrofolate reductase mutation is not associated with Alzheimer's disease. *Neurosci Lett* 2001; **315**: 103–105.
16 Wakutani Y, Kowa H, Kusumi M *et al.* Genetic analysis of vascular factors in Alzheimer's disease. *Ann N Y Acad Sci* 2002; **977**: 232–238.
17 Lievers KJ, Kluijtmans LA, Heil SG *et al.* A 31 bp VNTR in the cystathionine beta-synthase (CBS) gene is associated with reduced CBS activity and elevated post-load homocysteine levels. *Eur J Hum Genet* 2001; **9**: 583–589.
18 Kraus JP, Williamson CL, Figaira FA *et al.* Cloning and screening with nanogram amounts of immunopurified mRNAs: cDNA cloning and chromosomal mapping of cystathionine beta synthase and the beta subunit of propionyl-CoA carboxylase. *Proc Natl Acad Sci* 1986; **83**: 2047–2051.
19 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical Diagnosis of Alzheimer's Disease. report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology* 1984; **34**: 939–944.
20 Wenham PR, Price WH, Blundell G. Apolipoprotein E genotyping by one-stage PCR. *Lancet* 1991; **337**: 1158–1159.
21 Frosst P, Blom HJ, Milos R *et al.* A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nature Genet* 1995; **10**: 111–113.
22 Yang F, Hanson NQ, Schwichtenberg K, Tsai MY. Variable number tandem repeat in exon/intron border of the cystathionine beta-synthase gene: a single nucleotide substitution in the second repeat prevents multiple alternate splicing. *Am J Med Genet* 2000; **95**: 385–390.
23 Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatoraphy with fluorescence detection. *J Chromatogr* 1987; **422**: 43–52.
24 Hoshino T, Kamino K, Matsumoto M. Gene dose effect of the APOE-epsilon4 allele on plasma HDL cholesterol level in patients with Alzheimer's disease. *Neurobiol Aging* 2002; **23**: 41–45.
25 Afman LA, Lievers KJ, Kluijtmans LA, Trijbels FJ, Blom HJ. Gene–gene interaction between the cystathionine beta-synthase 31 base pair variable number of tandem repeats and the methylenetetrahydrofolate reductase 677C>T polymorphism on homocysteine levels and risk for neural tube defects. *Mol Genet Metab* 2003; **78**: 211–215.
26 Ueland PM, Refsum H, Beresford SA, Vollset SE. The controversy over homocysteine and cardiovascular risk. *Am J Clin Nutr* 2000; **72**: 324–332.
27 Sparks DL, Martin TA, Gross DR, Hunsaker JC 3rd. Link between heart disease, cholesterol, and Alzheimer's disease: a review. *Microsc Res Tech* 2000; **50**: 287–290.
28 Lehmann M, Gottfries CG, Regland B. Identification of cognitive impairment in the elderly: homocysteine is an early marker. *Dement Geriatr Cogn Disord* 1999; **10**: 12–20.
29 Morris MS, Jacques PF, Rosenberg IH, Selhub J. National Health and Nutrition Examination Survey. Hyperhomocysteinemia associated with poor recall in the third National Health and Nutrition Examination Survey. *Am J Clin Nutr* 2001; **73**: 927–933.

30 Ravaglia G, Forti P, Maioli F *et al.* Blood homocysteine and vitamin B levels are not associated with cognitive skills in healthy normally ageing subjects. *J Nutr Health Aging* 2000; **4**: 218–222.
31 Kalmijn S, Launer LJ, Lindemans J, Bots ML, Hofman A, Breteler MM. Total homocysteine and cognitive decline in a community-based sample of elderly subjects: the Rotterdam Study. *Am J Epidemiol* 1999; **150**: 283–289.
32 Williams JH, Pereira EA, Budge MM, Bradley KM. Minimal hippocampal width relates to plasma homocysteine in community-dwelling older people. *Age Ageing* 2002; **31**: 440–444.
33 den Heijer T, Vermeer SE, Clarke R *et al.* Homocysteine and brain atrophy on MRI of non-demented elderly. *Brain* 2003; **126**: 170–175.
34 Hogervorst E, Ribeiro HM, Molyneux A, Budge M, Smith AD. Plasma homocysteine levels, cerebrovascular risk factors, and cerebral white matter changes (leukoaraiosis) in patients with Alzheimer disease. *Arch Neurol* 2002; **59**: 787–793.
35 Postiglione A, Milan G, Ruocco A, Gallotta G, Guiotto G, Di Minno G. Plasma folate, vitamin B (12), and total homocysteine and homozygosity for the C677T mutation of the 5,10-methylene tetrahydrofolate reductase gene in patients with Alzheimer's dementia. A case-control study. *Gerontology* 2001; **47**: 324–329.
36 Mok SS, Turner BJ, Beyreuther K, Masters CL, Barrow CJ, Small DH. Toxicity of substrate-bound amyloid peptides on vascular smooth muscle cells is enhanced by homocysteine. *Eur J Biochem* 2002; **269**: 3014–3022.
37 de la Torre JC. Cerebral hypoperfusion, capillary degeneration, and development of Alzheimer disease. *Alzheimer Dis Assoc Disord* 2000; **14**: S72–S81.
38 Munke M, Kraus JP, Ohura T, Francke U. The gene for cystathionine beta-synthase (CBS) maps to the subtelomeric region on human chromosome 21q and to proximal mouse chromosome 17. *Am J Hum Genet* 1988; **42**: 550–559.
39 Korenberg J, Kawashima H, Pulst S *et al.* Molecular definition of the region of chromosome 21 that causes features of the Down syndromephenotype. *Am J Hum Genet* 1990; **47**: 236–246.
40 Wisniewski KE, Wisniewski HM, Wen GY. Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann Neurol* 1985; **17**: 278–282.
41 Eto K, Asada T, Arima K, Makifuchi T, Kimura H. Brain hydrogen sulfide is severely decreased in Alzheimer's disease. *Biochem Biophys Res Commun* 2002; **293**: 1485–1488.
42 Beyer K, Lao JI, Latorre P *et al.* Methionine synthase polymorphism is a risk factor for Alzheimer disease. *Neuroreport* 2003; **14**: 1391–1394.

Takeda M, Tanaka T, Cacabelos R (eds): Molecular Neurobiology of Alzheimer Disease and Related Disorders. Basel, Karger, 2004, pp 31–41

Alzheimer's γ-Secretase Mechanism Produces Amyloid-β-Protein Like Peptides Simultaneously with Release of Intracellular Signaling Fragments

Masayasu Okochi, Akio Fukumori, Yumi Satoh, Nuripa Aidaralieva, Hisashi Tanii, Kojin Kamino, Toshihisa Tanaka, Takashi Kudo, Masatoshi Takeda

Department of Post-Genomics and Diseases, Division of Psychiatry and Behavioral Proteomics, Osaka University Graduate School of Medicine, Osaka, Japan

The amyloid hypothesis posits that the process by which secreted soluble amyloid β-protein (Aβ) turns into its aggregated insoluble form is essential for the development of Alzheimer's disease (AD). This has been the leading hypotheses to explain the pathogenesis of AD [1]. Aβ, originally identified biochemically and always present as insoluble amyloid fibrils in senile plaques of AD brains, were found to be released physiologically from cells in the form of soluble peptides [2, 3]. The amyloid hypothesis was a logical solution of this contradiction. Senile plaques and neurofibrillary tangles (NFT) are pathological structures characteristic of AD. However, although senile plaques are AD specific, NFT occur as broader and more general lesions in neurodegenerative diseases [4]. This indicates that senile plaques are related to the AD-specific pathological process, whereas NFT are more closely related to general processes of neurodegeneration.

Aβ is generated by sequential cleavages of the β-amyloid precursor protein (βAPP) [5]. Causative mutations for familial AD have been identified in *presenilins (PSs)* and *βAPP* genes [6]. It has been proposed that PS, as functions of these genes, are proteolytic enzymes (γ-secretases) and βAPP is thought to be one of their substrates [7], which is consistent with the fact that proteolytic cleavage (γ-cleavage) is directly responsible for Aβ generation [5].

These findings emphasize the importance of the Aβ peptide for understanding the pathological process of AD.

Except for unusual conditions, all pathological AD mutants of PS and βAPP affect the precision of the γ-cleavage site of βAPP [1], that is, the mutants cause a partial shift of the γ-cleavage site in the direction of the C-terminal with 2–3 amino acids [1]. As a result, the generative ratio of Aβ species ending 42 (Aβ numbering) in relation to that of the major Aβ species, ending 40 is upregulated [8]. Because (1) fibrillization of Aβ42 is much faster than that of Aβ40, and (2) Aβ42 is the major accumulating Aβ species in AD, the relative upregulation of Aβ42 in familial AD plays a central role in the insolubilization and accumulation of Aβ in the brain [1]. Aβ42 deposition in SP is also an invariant phenotype of sporadic AD, and is observed in the majority of AD cases. However, because of the highly aggregative nature of Aβ42, it has been very difficult to determine whether the precision of the γ-cleavage is affected, and thus whether Aβ42 generation is upregulated in sporadic AD brains. Nevertheless, Aβ42 peptide could be not only, as seen earlier, a substance which regulates the AD pathological process, but theoretically also one of the most effective biomarkers for AD. However, again because of its extremely aggregative nature, the Aβ42 level in CSF or peripherally of AD patients usually decreases and does not reflect its generation [9], which makes it difficult to use this level as a prediagnostic marker of AD.

We have recently found that a group of peptides may be secreted by the same mechanism as that for γ-secretase of βAPP [10, 11]. We also found that the precision of this cleavage is affected by familial AD-associated PS1 mutations similar to the pathological endoproteolysis of βAPP [10]. Therefore, by measuring these Aβ-like peptides instead of Aβ, it may be possible to determine whether γ-cleavage of βAPP is affected in sporadic AD brain. Further, it is theoretically possible that this might lead to the use of peptide levels as a prediagnostic marker for AD.

Intramembranous Endoproteolysis Is Essential for the Novel Signaling Paradigm

It is well known that signal transduction plays an important role in neural functions. In its classical form, the signal transduction paradigm is understood to mean that ligand binding to cell surface receptors induces activation of intracellular kinases or ion influx into cytosol, which functions as a second messenger. It is true that this simple paradigm has helped to explain a number of signaling events. Recently, however, a novel signaling mechanism has been proposed, in which membrane-anchored cell surface receptors themselves

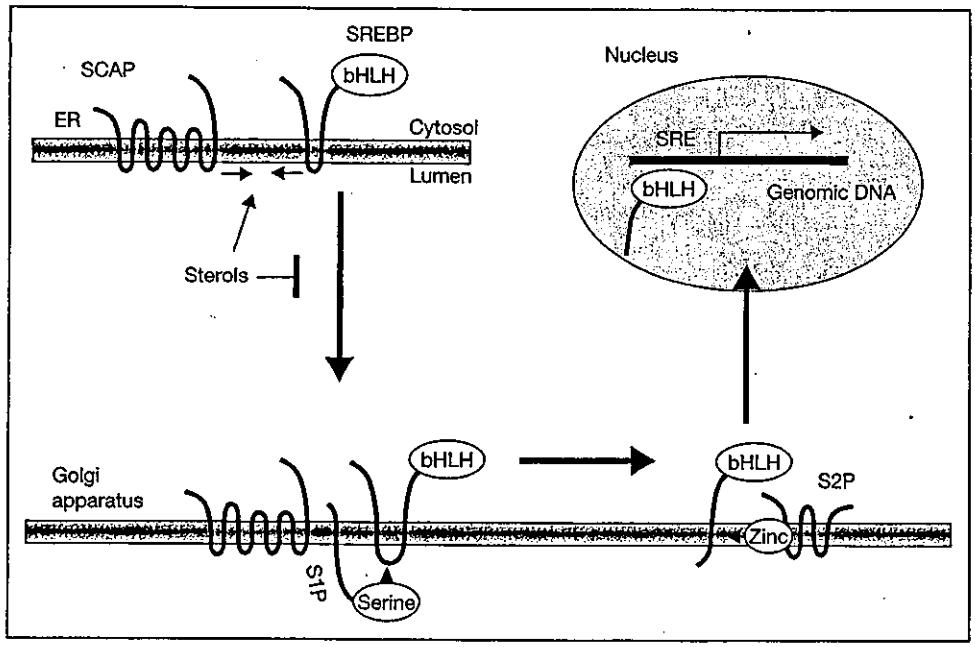

Fig. 1. Endoproteolysis of SREBPs: Concept of RIP. Serine of S1P indicates a proteolytic active center and zinc of S2P metal ion binding to the active center.

undergo sequential endoproteolysis upon ligand binding, and their intracellular domains directly translocate to the nucleus and function as transcription modifiers [12, 13].

The biochemical characteristic of such a signaling mechanism is the importance of intramembranous endoproteolysis which releases the cytosolic domain of the receptor from membranes [12, 13]. That is, fragments which translocate to the nucleus and modify transcription are immediately generated by a special form of intramembranous endoproteolysis. This cleavage is known as regulated intramembranous proteolysis (RIP) [13]. RIP is an as yet largely unknown endoproteolysis which can hydrolyse a peptide bond in a highly hydrophobic environment. RIP was first described in connection with the sequential endoproteolysis of sterol regulatory element-binding protein (SREBP) (fig. 1) [13], a membrane-bound transcription factor which regulates cholesterol homeostasis. SREBP cleavage-activating protein (SCAP), a sensor for intracellular sterols, recognizes a reduction in sterols and transports SREBPs from the endoplasmic reticulum (ER) to the Golgi membrane. The transported SREBPs are then sequentially cleaved by two Golgi-resident membrane proteases, site-1 protease (S1P) and site-2 protease (S2P), which release from the membrane the basic helix-loop-helix-leucine zipper (bHLH-Zip)

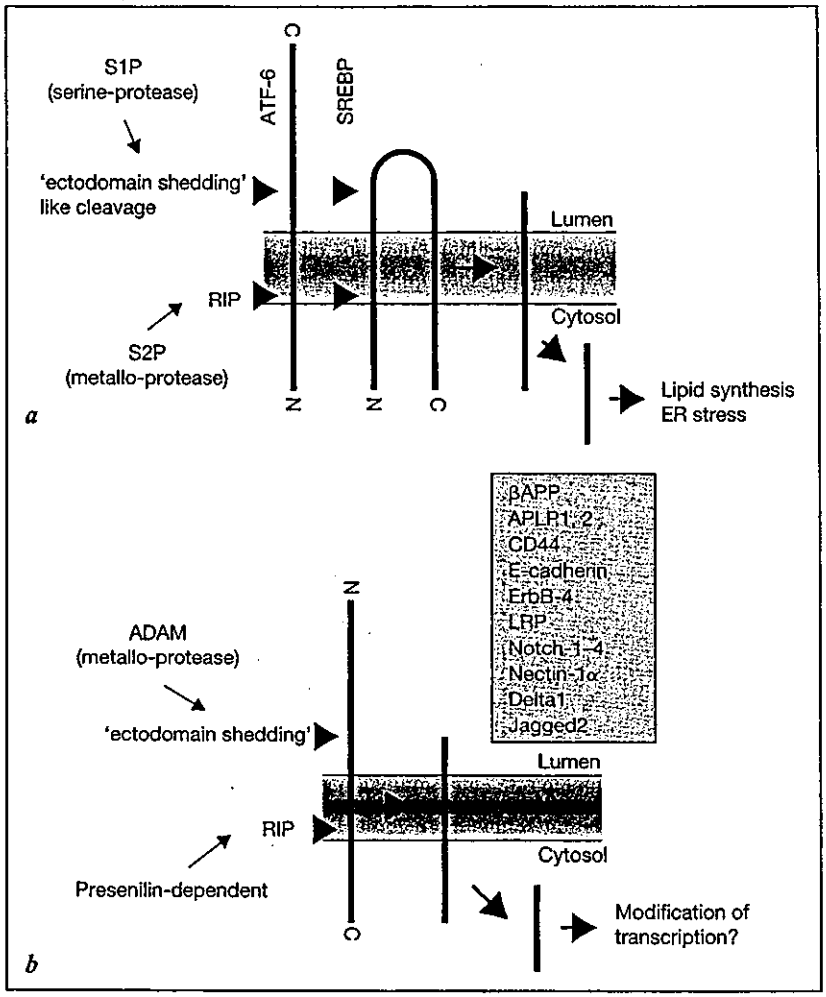

Fig. 2. ***a*** Sequential cleavages of ATF-6 and SREBPs share common features. ***b*** Common sequential cleavage mechanism (RIP) when substrates share the type-1 topology in their transmembrane domain.

domain as NTF. The functional bHLH-Zip domain then translocates to the nucleus and binds to the sterol regulatory element (SRE), which resides in the enhancer or promoter region of the target genes. When the intracellular cholesterol level increases, generation of the SCAP/SREBP complex is eliminated, which then inhibits release of the bHLH-Zip domain from the membrane and is followed by a decrease in the transcription of all target genes.

Recently, a type II membrane-anchored transcriptional factor ATF6, which is activated in ER stress response, has been shown to be a substrate for the sequential cleavages by S1P and S2P (fig. 2a) [14]. Striking similarities in

Table 1. List of putative polytopic TM I-Clips

protease	class	TM topology	substrates
S2P family	Metallo	type-2	SREBP, ATF6
Rhomboid family	Serine	type-1	TGF-α
Presenilins	Aspartic	type-1	APP, Notch, CD44, Erb-B4, etc.
SPP family	Aspartic	type-2	Signal peptide remnants

All proteases identified so far are soluble or single TM proteins, whereas all candidates for I-Clips are putative polytopic TM proteins. Moreover, I-Clips generally contain their proteolytic active centers in the hydrophobic sequence. These emphasize unusual characteristics of I-Clips. Amino acid sequences around active centers of I-Clips are reportedly not similar to conventional proteases but, in some cases, almost identical between I-Clips, which indicates that some unknown common mechanism might underlie this mysterious proteolysis.

endoproteolysis of ATF6 and SREBP can easily be found. In both cases, 'ectodomain shedding' by S1P triggers intramembranous endoproteolysis by S2P, which in turn generates NTF that translocate to the nucleus [15]. Induction of GRP78, an ER chaperone, is eliminated in cells lacking S2P [14]. Interestingly, when both S1P and S2P are involved in RIP, the transmembrane domain of the substrates seems to share type II topology.

On the other hand, when a disintegrin and metallo-protease (ADAM)- and PS-dependent γ-secretase mechanism is involved in RIP, the transmembrane domains of the substrate receptors appear to have a type I topology (fig. 2b). In addition to Notch receptors [16, 17], βAPP [18], ErbB-4 [19], E-cadherin [20], LRP [21], CD44 [11, 22], nectin-1α [23], Delta1 [31], and Jagged2 [31] have so far been identified as substrates for this mechanism. Although still controversial, these proteins are basically thought to undergo 'extracellular shedding' which is a prerequisite for consecutive PS-dependent proteolysis. Intramembrane cleaving proteases (I-Clips) are summarized in table 1.

PS comprise eight potent transmembrane proteins with both an N- and a C-terminus in cytosol [24] and occurring in high molecular weight complexes (~500 kD) [25]. PS produce γ-secretase activity, which generates both the C-terminus of Aβ and the N-terminus of the βAPP intracellular cytoplasmic domain (AICD) [26]. Genomic knock-out of PS1 or PS1/2 causes Notch phenotype in vivo, which shows that the major function of PS is to mediate Notch signaling [27]. Notch signaling was found to be a common signaling mechanism for metazoans which plays an essential role in neural differentiation from ectoderm [12]. Recently, however, this signaling has been found to play

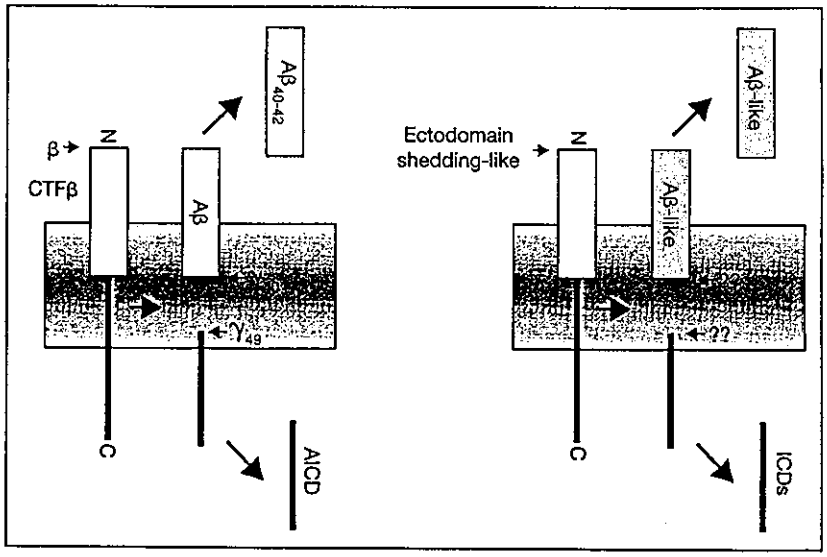

Fig. 3. Aβ-like peptides are secreted through signal transduction mediated by PS-dependent RIP.

various roles not only during development but also in adulthood. Notch signaling is realized only when Notch ligands (DSL family proteins) expressed in signaling cells bind to Notch receptors expressed in the signal-receiving cells. Upon binding to ligands, Notch receptors undergo sequential endoproteolysis, which results in the release of the cytosolic C-terminal fragment, NICD (Notch intracellular cytoplasmic domain), which is believed to directly translocate to the nucleus and regulate transcription of target genes [12].

Notch-1-β and CD44-β Peptides, Aβ-Like Fragments, Are Physiologically Secreted

We have analyzed in detail the PS-dependent intramembranous proteolysis of Notch-1 [10] and CD44 [11] and found that, as a result of the endoproteolysis, the Aβ-like Notch (Notch-1 Aβ-like peptide: Nβ) or CD44 (CD44 Aβ-like peptide: CD44β) fragment was extracellularly secreted as NTF [10, 11] (fig. 3). This indicates that at least several peptides that contain a transmembrane domain-like Aβ are secreted in vivo (fig. 4a). We suggest that secretion of peptides containing the transmembrane domain may be a phenomenon common to all substrates for PS-dependent endoproteolysis. Interestingly, the C-termini of these secreted peptides do not directly correspond to the N-termini of cytosolic C-terminal fragments (CTFs) functioning as signaling molecules,

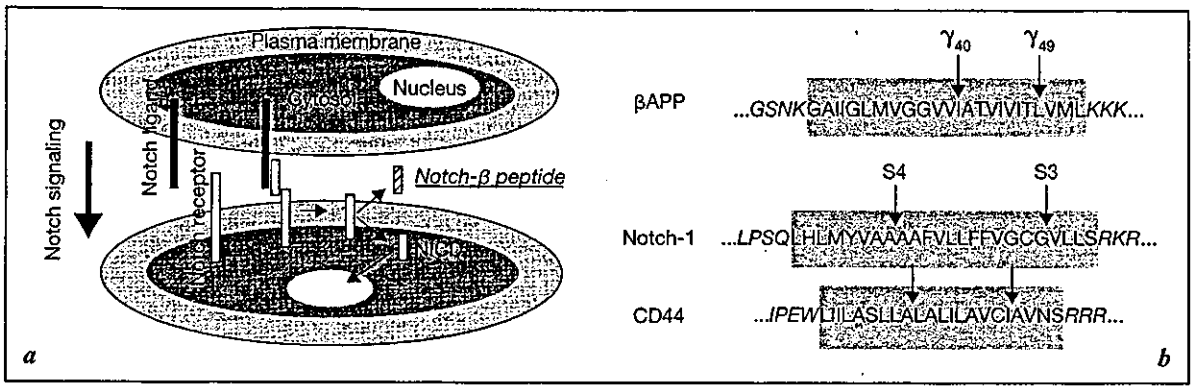

Fig. 4. ***a*** Notch signaling accompanies secretion of Notch-β peptide. By sequential endoproteolysis of Notch-1 shown in figures 2b, 3, an Aβ-like peptide, Nβ, is released. ***b*** PS-dependent intramembranous proteolysis, which we termed the 'dual cleavage' mechanism. Arrows indicate proteolytic cleavage sites. Small transmembrane peptides between 2 cleavage sites (arrows) have not yet been identified.

but are formed by distinct proteolysis upstream of N-termini of CTFs (fig. 4b). Thus, intramembranous endoproteolysis, which liberates an Aβ-like peptide, essentially consists of a distinct dual endoproteolysis, which we have termed 'dual cleavage' mechanism (fig. 4b) [10, 11]. These findings seem to indicate that 'dual cleavage' is necessary to degrade and liberate transmembrane peptides from membrane.

An important finding is that, similar to the pathological cut of βAPP, the precision of the γ-cleavage-generating C-terminus of Nβ is affected by familial-AD-associated PS1/2 mutations (fig. 5) [10]. These mutations were found to cause a partial shift in the cleavage site that generates increased levels of Nβ species whose C-termini are elongated by 2–4 amino acids [10]. This means that the level of secretion of Nβ1733-35 compared to that of Nβ1731, the most abundant Nβ species, is upregulated in the mutant-expressing cells [10]. We therefore suggest that secretion of Aβ-like peptides such as Nβ share the same γ-secretase mechanism as that of Aβ (see also fig. 2b, 3, 4b, 5).

Level of an Elongated Aβ-Like Peptide as a Substitute for Aβ May Reflect AD-Associated Pathological Impairment of γ-Secretase

Although the findings are only preliminary, Nβ, like Aβ, did not seem to aggregate, fibrillate nor accumulate in AD brains [Okochi and Arai,

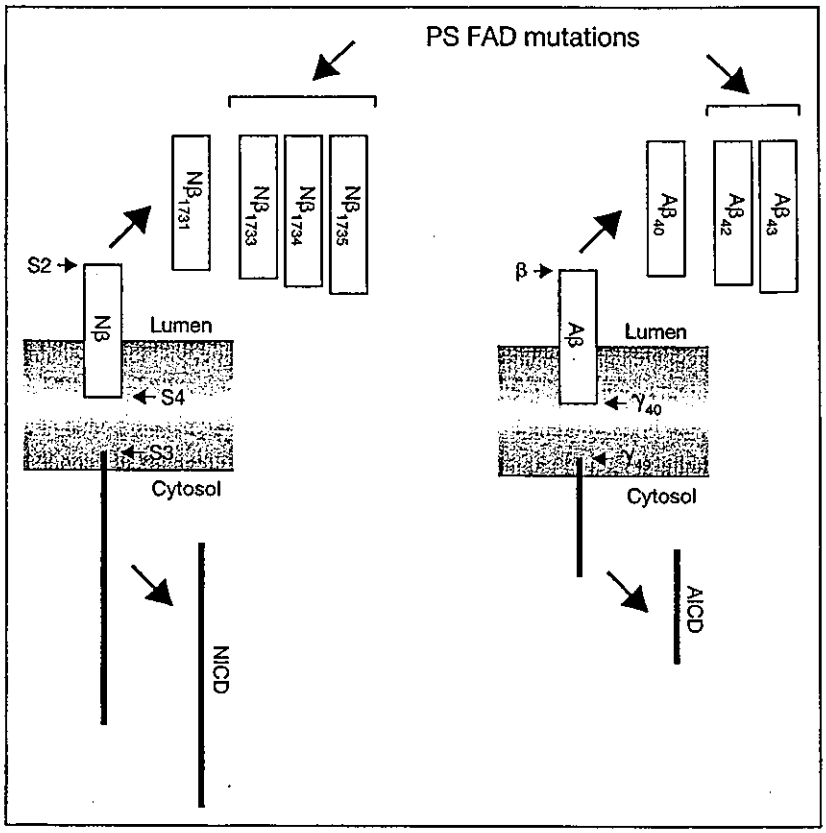

Fig. 5. Familial-AD-associated PS mutations affect γ-cleavage and elongate Nβ. These mutants showed a very similar effect on the precision of the γ-cleavage for the two distinct substrates. The magnitude of the effect, as analyzed so far, was not dependent on the substrates but on mutations of PS. In other words, PS mutants, while dramatically upregulating Aβ42 generation, simultaneously increase the level of elongated Nβ. This seems to indicate that mutations affect direct interaction between PS and their substrates.

unpubl. obs.]. Therefore, by measuring Nβ or the level of Nβ1733-35 relative to that of Nβ1731 in CSF or peripheral, it may be possible to determine the level of γ-secretase activity or Aβ42/40 generation ratio in patients with sporadic AD (see also fig. 5). Aβ deposition leading to AD may gradually take place over a number of years. It is likely that, in the process, γ-secretase activity is upregulated [28] or the precision of γ-cleavage in the brain is affected [1]. Therefore, by measuring the level of Aβ-like peptide or the relative level of elongated species in healthy individuals, it may be possible to diagnose those who are likely to develop AD before they show symptoms.

It Is Worthwhile Studying Whether Secreted Nβ Peptide Level Is Upregulated in Cells of Human Malignancies

Various kinds of examinations have demonstrated that expression levels of Notch receptors including Notch-1 are strongly upregulated in tumor cells, which indicates that Notch signaling is promoted in human malignancies [29]. Very recent evidence indicates a novel mode of cross-talk between the epidermal growth factor receptor/Ras/mitogen-activated protein kinase cascade and the Notch pathway [30]. Moreover, there are indications that oncogenic mutants of Ras observed in 25–50% of human cancer perform an oncogenic function through Notch signaling [29]. One might therefore argue that Notch signaling, a local cell signaling which suppresses cell differentiation and promotes proliferation, may be involved in tumor genesis itself. However, since no one knows how local cell signaling can be monitored, no attempts have been made so far, to measure the signaling level or to evaluate the level in relation to diagnosis or therapy for human tumor in vivo. We have discovered that, for each Notch-signaling fragment produced, a kind of 'peptide evidence' of the signaling event is definitely secreted extracellularly (see also fig. 4a). By measuring this Nβ peptide level, therefore, the level of Notch signaling may be assessed.

Acknowledgement

We wish to thank Dr. Tetsuaki Arai of the Department of Neuropathology, Tokyo Institute of Psychiatry, for pathological characterization of anti-Nβ antibodies and our colleagues at the Laboratory for Biochemistry, especially Dr. Naohiko Matsumoto, for critically reading the manuscript. This research project has been conducted in close collaboration with Prof. Christian Haass and Dr. Harald Steiner of the Department of Biochemistry, Laboratory for Alzheimer's Disease Research, Ludwig Maximilian University, Munich. This work was supported by grants from the Ministry of Education, Science, Culture and Sports (14017060 and 14770499 to M.O.) and the Ministry of Health and Welfare (14121601 to M.T. and M.O. and 13080101 to M.T.).

References

1 Selkoe DJ: Alzheimer's disease: Genes, proteins, and therapy (review). Physiol Rev 2001;741–766.

2 Haass C, Schlossmacher MG, Hung AY, Vigo-pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB, et al: Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature 1992;359:322–325.

3 Shoji M, Golde TE, Ghiso J, Cheung TT, Estus S, Shaffer LM, Cai XD, McKay DM, Tintner R, Frangione B, et al: Production of the Alzheimer amyloid beta protein by normal proteolytic processing. Science 1992;258:126–129.

4 Hardy J: Genetic dissection of primary neurodegenerative diseases (review). Biochem Soc Symp 2001;67:51–57.

5 Haass C, Selkoe DJ: Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide (review). Cell 1993;75:1039–1042.
6 St George-Hyslop PH: Molecular genetics of Alzheimer's disease review. Biol Psychiatry 2000; 47:183–199.
7 Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ: Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 1999;398:513–517.
8 Steiner H, Haass C: Intramembrane proteolysis by presenilins (review). Nat Rev Mol Cell Biol 2000;1:217–224.
9 Teunissen CE, de Vente J, Steinbusch HW, De Bruijn C: Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid. Neurobiol Aging 2002;23:485–508.
10 Okochi M, Steiner H, Fukumori A, Tanii H, Tomita T, Tanaka T, Iwatsubo T, Kudo T, Takeda M, Haass C: Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. EMBO J 2002;21:5408–5416.
11 Lammich S, Okochi M, Takeda M, Kaether C, Capell A, Zimmer AK, Edbauer D, Walter J, Steiner H, Haass C: Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. J Biol Chem 2002;277:44754–44759.
12 Mumm JS, Kopan R: Notch signaling: From the outside in (review). Dev Biol 2000;228:151–165.
13 Brown MS, Ye J, Rawson RB, Goldstein JL: Regulated intramembrane proteolysis: A control mechanism conserved from bacteria to humans (review). Cell 2000;100:391–398.
14 Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R, Brown MS, Goldstein JL: ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell 2000;6:1355–1364.
15 Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K: XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107:881–891.
16 Mizutani T, Taniguchi Y, Aoki T, Hashimoto N, Honjo T: Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members. Proc Natl Acad Sci USA 2001;98:9026–9031.
17 Saxena MT, Schroeter EH, Mumm JS, Kopan R: Murine notch homologs (N1-4) undergo presenilin-dependent proteolysis. J Biol Chem 2001;276:40268–40273.
18 Naruse S, Thinakaran G, Luo JJ, Kusiak JW, Tomita T, Iwatsubo T, Qian X, Ginty DD, Price DL, Borchelt DR, Wong PC, Sisodia SS: Effects of PS1 deficiency on membrane protein trafficking in neurons. Neuron 1998;21:1213–1221.
19 Lee HJ, Jung KM, Huang YZ, Bennett LB, Lee JS, Mei L, Kim TW: Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. J Biol Chem 2002;277:6318–6323.
20 Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V, Baki L, Wen P, Efthimiopoulos S, Shao Z, Wisniewski T, Robakis NK: A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. EMBO J 2002;21:1948–1956.
21 May P, Reddy YK, Herz J: Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem 2002;277:18736–18743.
22 Okamoto I, Kawano Y, Murakami D, Sasayama T, Araki N, Miki T, Wong AJ, Saya H: Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. J Cell Biol 2001;155:755–762.
23 Kim DY, Ingano LA, Kovacs DM: Nectin-1alpha, an immunoglobulin-like receptor involved in the formation of synapses, is a substrate for presenilin/gamma-secretase-like cleavage. J Biol Chem 2002;277:49976–49981.
24 Li X, Greenwald I: Membrane topology of the *C. elegans* SEL-12 presenilin. Neuron 1996;17: 1015–1021.
25 Edbauer D, Winkler E, Haass C, Steiner H: Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. Proc Natl Acad Sci USA 2002;99: 8666–8671.
26 Haass C, Steiner H: Alzheimer disease gamma-secretase: A complex story of GxGD-type presenilin proteases. Trends Cell Biol 2002;12:556–562.

Okochi/Fukumori/Satoh/Aidaralieva/Tanii/Kamino/Tanaka/Kudo/Takeda

27 Wong PC, Zheng H, Chen H, Becher MW, Sirinathsinghji DJ, Trumbauer ME, Chen HY, Price DL, Van der Ploeg LH, Sisodia SS: Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. Nature 1997;387:288–292.
28 Ikeuchi T, Dolios G, Kim SH, Wang R, Sisodia SS: Familial Alzheimer disease-linked presenilin 1 variants enhance production of both Abeta 1–40 and Abeta 1–42 peptides that are only partially sensitive to a potent aspartyl protease transition-state inhibitor of 'gamma-secretase'. J Biol Chem 2003;278:7010–7018.
29 Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, Osborne BA, Gottipati S, Aster JC, Hahn WC, Rudolf M, Siziopikou K, Kast WM, Miele L: Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 2002;8:979–986.
30 Shaye DD, Greenwald I: Endocytosis-mediated downregulation of LIN-12/Notch upon Ras activation in *Caenorhabditis elegans*. Nature 2002;420:686–690.
31 Ikeuchi T, Sisodia SS: The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent "gamma-secretase" cleavage. J Biol Chem 2003;278:7751–7754.

Masayasu Okochi
Department of Post-Genomics and Diseases
Division of Psychiatry and Behavioral Proteomics
Osaka University Graduate School of Medicine, 565–0871 Osaka (Japan)
Tel. +81 6 6879 3053, Fax +81 6 6879 3059, E-Mail mokochi@psy.med.osaka-u.ac.jp

Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1031 (2005) 284–296
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research report

Accumulation of aluminum by primary cultured astrocytes from aluminum amino acid complex and its apoptotic effect

David A. Aremu, Shunsuke Meshitsuka*

Division of Integrative Bioscience, Department of Biomedical Science, Graduate School of Medical Science, Tottori University, 86 Nishi-machi, Yonago 683-8503, Japan.

Accepted 25 June 2004
Available online 16 December 2004

Abstract

Aluminum salts or doses that are unlikely in the human system have been employed in toxicity studies and much attention had been focused on the secondary target (neurons) of its toxicity rather than the primary target (astroglia). In order to address these issues, we have investigated the uptake and apoptotic effects of aluminum amino acid complex on primary cultured astrocytes because these are fundamental in understanding the mechanism of aluminum neurotoxicity. Aluminum solubilized by various amino acids was differentially internalized by astrocytes (glycine>serine≫glutamine≫glutamate), but aluminum was not internalized from citrate complex following 24 h of exposure. Inhibition of glutamine synthetase, by methionine sulfoximine (MSO), enhanced the uptake of aluminum from various amino acid complexes within 8 h except from glutamine complex. Blockade of selective GLT-1 (EAAT2) and GlyT1, as well as nonspecific transporters, did not inhibit or had no effect on uptake of aluminum in complex with the corresponding amino acids. Ouabain also failed to inhibit uptake of aluminum complexed with glycine. Pulse exposure to aluminum glycinate in the absence or presence of MSO caused apoptosis in over 25% of primary cultured astrocytes, and apoptotic features such as chromatin condensation and fragmentation became evident as early as 3 days of culture in normal medium. Lower doses (as low as 0.0125 mM) also caused apoptosis. The present findings demonstrate that aluminum solubilized by amino acids, particularly glycine, could serve as better candidate for neurotoxicity studies. Citrate may be a chelator of aluminum rather than a candidate for its cellular uptake. Amino acid transporters may not participate in the uptake of aluminum solubilized by their substrates. Another pathway of aluminum internalization may be implicated in addition to passive diffusion but may not require energy in form of Na^+/K^+-ATPase. Impaired astrocyes' metabolism can aggravate their accumulation of aluminum and aluminum can compromise astrocytes via apoptosis. Thus, loss of astrocytic regulatory and supportive roles in the central nervous system (CNS) may be responsible for neurodegeneration observed in Alzheimer's disease.

Theme: Disorders of the nervous system
Topic: Neurotoxicology

Keywords: Aluminum amino acid complex; Internalized aluminum; Metabolic perturbation; Amino acid transporter; Apoptosis; Astroglial culture

1. Introduction

The recognition of aluminum as a neurotoxic agent in animal dates back to over 100 years [25], but the idea that aluminum may be involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD), was first suggested about four decades ago in the report of Klatzo et al. [39]. About a decade following this report, Crapper et al. [18] corroborated the findings of Klatzo et al. Alfrey et al. [5] also reported their findings, implicating aluminum in the etiology of dialysis encephalopathy. Since these times, the public has been besieged by conflicting reports supporting, refuting, or equivocal on those claims. Aluminum has also been implicated in several other neurological and non-neurological disorders. However, the existence of causal relationship between aluminum and neurodegenerative disorders, such as AD, remains

* Corresponding author. Fax: +81 859 34 8068.
E-mail address: mesh@grape.med.tottori-u.ac.jp (S. Meshitsuka).

0006-8993/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2004.06.090