Table 1. Association of SNP0070 in *SNCA* between cases and controls

	Genotype				Allele			*P*-value (χ^2-test)			
	CC	CT	TT	Total	C	T	Total	Genotype	Allele	Dominant[a] model	Recessive[b] model
First screen											
Case	87 (0.46)	87 (0.46)	14 (0.07)	188	261 (0.69)	115 (0.31)	376	3.4×10^{-4}	1.8×10^{-4}	1.8×10^{-4}	1.1×10^{-2}
Control	62 (0.33)	85 (0.46)	39 (0.21)	186	209 (0.56)	163 (0.44)	372				
Replication											
Case	298 (0.44)	307 (0.45)	75 (0.11)	680	903 (0.66)	457 (0.34)	1360	1.3×10^{-6}	4.2×10^{-7}	1.5×10^{-3}	9.0×10^{-7}
Control	233 (0.31)	387 (0.52)	126 (0.17)	746	853 (0.57)	639 (0.43)	1492				
Total											
Case	385 (0.44)	394 (0.45)	89 (0.10)	868	1164 (0.67)	572 (0.33)	1736	2.7×10^{-9}	5.0×10^{-10}	5.7×10^{-6}	2.8×10^{-8}
Control	295 (0.32)	472 (0.51)	165 (0.18)	932	1062 (0.57)	802 (0.43)	1864				

Frequencies of genotypes and alleles are in parentheses.
[a]Genotype CC+CT versus TT.
[b]Genotype CC versus CT+TT.

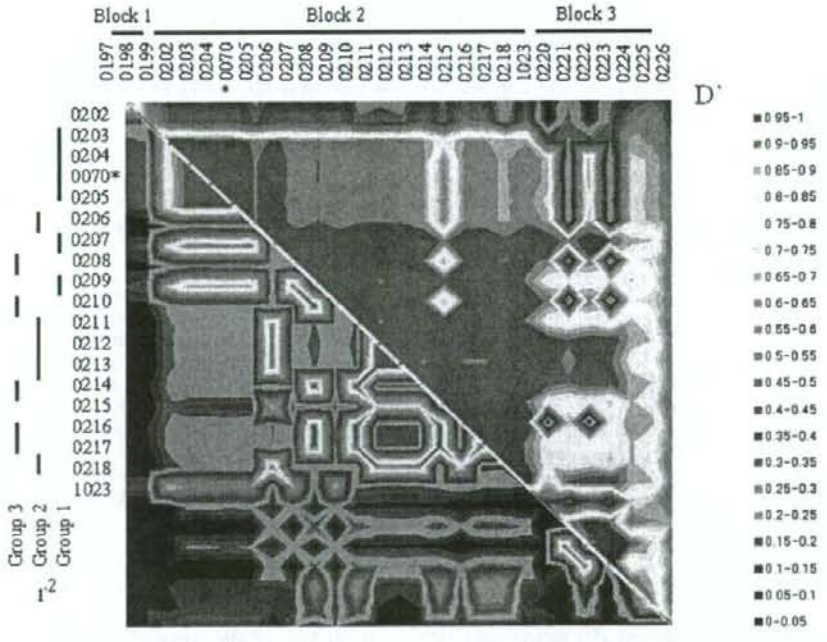

Figure 1. LD structure of the susceptibility region for sporadic PD. Pairwise LD between SNPs, as measured by D' in 134 controls, is graphically indicated. The region spanning 430 kb around the originally screened SNP0070(*) was divided into three LD blocks ($D' > 0.9$) (upper right). On the basis of r^2, SNPs in block 2, including SNP0070, were further divided into three groups ($r^2 > 0.85$) and three solitary SNPs (lower left). The scale is nominal.

SNCA gene expression in relation to susceptibility genotypes

To examine whether the strongest associated SNPs (group 1) affect *SNCA* gene expression, we further quantified *SNCA* mRNA in autopsied frontal cortices and compared the values among the genotypes. SNP0070, in which allele C is associated with PD, was used as a representative of group 1.

The relative values of *SNCA* mRNA for all cases ($n = 21$) and all controls ($n = 18$) were 1.07 ± 0.10 and 0.95 ± 0.13, respectively, showing almost the same level ($P = 0.46$, Student's *t*-test). When compared among the genotypes in cases, the mean tended to decrease in the order of CC, CT and TT (Fig. 3), although the differences did not reach the significant levels ($P = 0.71$ for CC versus CT, $P = 0.16$ for CT versus TT and $P = 0.32$ for CC versus TT). Similar tendency

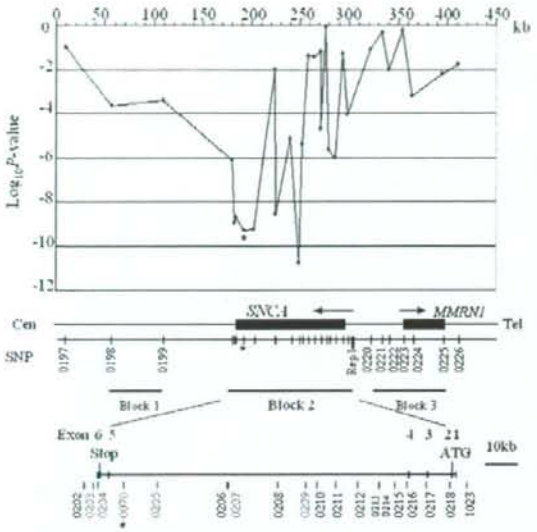

Figure 2. Genomic structure and SNPs of the susceptibility region for sporadic PD and case–control association studies (882 cases and 938 controls). Log *P*-values (allele 1 versus allele 2) are plotted against the physical location of the SNPs. The region includes two genes: *SNCA* and *MMRN1*; transcription orientation is indicated by horizontal arrows. Physical locations of SNPs are shown as axial bars with our experimental ID number. The originally screened SNP0070 is indicated by an asterisk. The location of Rep1, a well-known repeat polymorphism in the *SNCA* promoter region, is indicated by a thick bar. SNPs in block 2 are nominated in an expanded map with the exon–intron structure of *SNCA*. SNPs in group 1 are shown in red. Note that *P*-values are prominently low at the group 1 SNPs located in the 3′ region of *SNCA*. *P*-values in the region around Rep1 are far from significant when compared with those in group 1.

was observed in controls. The mean tended to decrease in the order of CC, CT and TT (Fig. 3) ($P = 0.33$ for CC versus CT, $P = 0.59$ for CT versus TT and $P = 0.54$ for CC versus TT).

These results indicate the possibility that expression of *SNCA* mRNA in the brain tends to be positively correlated with the number of PD-associated allele.

DISCUSSION

To identify susceptibility genes for PD, we performed an extensive candidate gene approach by screening 268 SNPs in 121 genes and identified a prominent association with SNP0070 (rs7684318) in the *SNCA* gene (Table 1). LD mapping localized the entire *SNCA* gene within a single LD block (Figs 1 and 2). Within this block, six SNPs including SNP0070 were in a tight LD group and most strongly associated with PD (Fig. 2; Table 2). The major allele of each SNP in group 1 was positively associated with PD, more strongly in the recessive model than in the dominant model (Table 4). Our genetic analyses establish *SNCA* as a definite susceptibility gene for PD and identify multiple SNPs in group 1 as susceptibility SNPs. Recently, Mueller *et al.* (23) reported that multiple regions of *SNCA* are associated with PD in the German population. Associated SNPs identified by Mueller *et al.* included rs356165 ($P = 1.5 \times 10^{-4}$), which corresponds to SNP0204 in our study, indicating that this SNP has a similar association in Caucasians. Pals *et al.* (24) previously reported no association of the haplotype containing rs356165 with PD in Belgian samples. This contradictory finding may be, at least in part, due to a small sample size (175 cases and 186 controls), as mentioned by the authors.

SNCA/α-synuclein was originally identified in the electric organ of the Pacific electric ray (25). SNCA is a presynaptic protein that is highly and broadly expressed in the brain, but its normal function remains unknown (21). It is a major component of Lewy bodies, the pathological hallmark of PD (20), and the aggregation of SNCA protein is thought to play a crucial role in the loss of dopaminergic neurons (21,26).

SNCA was also the first gene identified as a causative gene in familial PD. Three missense mutations in *SNCA* were reported in families with AD inheritance (6,27,28). These mutations are thought to increase the aggregation of SNCA protein. Point mutations in *SNCA* have not been identified in sporadic PD (27,29), and no SNPs have been found in the coding region, suggesting that disease-related amino acid changes in SNCA are unlikely in sporadic PD.

Genes' overdosage is a potential mechanism for the influence of *SNCA* in PD. Triplication of the *SNCA* locus has been seen in an AD PD family (30), and doubling of *SNCA* gene dosage by triplication has been shown to result in the doubling of mRNA and protein expression in blood and brain (31). Duplication of *SNCA* has also been identified as a cause of familial PD (32,33). Clinical features of patients with *SNCA* duplication resemble those of sporadic cases and are much milder than those with triplication. Taken together, these observations indicate a correlation between increased SNCA protein levels and disease risk. Identification of one or more polymorphisms related to *SNCA* expression level might reveal strong susceptibility indicators for sporadic PD. Many studies have focussed on a mixed repeat microsatellite polymorphism called Rep1 (34), because of its location in the *SNCA* promoter region. However, their significance is uncertain, possibly because of the small number of samples (35–37). Our study demonstrates that the *P*-values of SNPs around Rep1 (0218, 1023 and 0220) are less significant than that of the SNPs in group 1 (Fig. 2). In addition, we genotyped our samples for Rep1. Pairwise D'-values showed that Rep1 was not in block 2, but on the boundary (Supplementary Material, Table S2). *P*-value of Rep1 was 7.5×10^{-7} (Supplementary Material, Table S3), which might be explained by its intermediate correlation with the strongest susceptibility SNPs (group 1, $P = 2.0 \times 10^{-9}$–1.7×10^{-11}). Our findings suggest that *P*-value of Rep1 depends on its LD strength with SNPs in group 1. LD strength may be modified by the unstableness of microsatellite markers (38) and may vary among races (39). Taken together, these findings may also partly explain the contradictory findings of previous Rep1 association studies.

To investigate the relationship between the SNPs in group 1 and the *SNCA* expression levels, we analyzed *SNCA* mRNA expression in autopsied frontal cortices (Fig. 3). *SNCA* expression levels tended to be positively correlated with the number of the PD-associated allele, supporting the popular hypothesis that increased SNCA leads to the disease.

Table 2. Association analysis in *SNCA* and surrounding region

SNPs			LD block	Genotype							
ID (rs ID)	Alleles 12	Location	(group)	Case 11/12/22	(Total)	Control 11/12/22	(Total)	MAF Case/control	Allele 1 versus allele 2 *P*-value	OR (95% CI)	HWE Case/control
0197 (rs3733450)	TC		1	38/286/549	(873)	33/280/619	(932)	0.21/0.19	0.10	1.15 (0.97–1.36)	1.00/0.93
0198 (rs1390280)	AG		1	366/384/118	(868)	316/454/162	(932)	0.36/0.42	2.1×10^{-4}	1.29 (1.13–1.46)	0.32/1.00
0199 (rs3733449)	CT		1	117/375/374	(866)	154/451/322	(927)	0.35/0.41	3.7×10^{-4}	1.28 (1.11–1.48)	0.16/0.91
0202 (rs356221)	TA	3′-flanking	2	73/369/431	(873)	123/449/360	(932)	0.30/0.37	7.2×10^{-7}	1.42 (1.25–1.63)	0.69/0.40
0203 (rs3857053)	TC	3′-flanking	2 (1)	380/406/87	(873)	293/476/164	(933)	0.33/0.43	1.1×10^{-9}	1.53 (1.33–1.73)	0.18/0.24
0204 (rs356165)	GA	3′-UTR	2 (1)	379/399/89	(867)	289/482/159	(930)	0.33/0.43	2.0×10^{-9}	1.52 (1.33–1.74)	0.32/0.09
0070[a] (rs7684318)	CT	Intron 4	2 (1)	385/394/89	(868)	295/472/165	(932)	0.33/0.43	5.0×10^{-10}	1.54 (1.35–1.75)	0.47/0.35
0205 (rs3775424)	CT	Intron 4	2 (1)	87/406/376	(869)	166/477/288	(931)	0.33/0.43	5.4×10^{-10}	1.52 (1.34–1.75)	0.16/0.22
0206 (rs3775426)	CT	Intron 4	2 (2)	56/350/456	(862)	53/324/555	(932)	0.27/0.23	0.0098	1.22 (1.05–1.41)	0.35/0.59
0207 (rs3796661)	CT	Intron 4	2 (1)	91/367/382	(840)	154/482/296	(932)	0.33/0.42	2.7×10^{-4}	1.52 (1.31–1.76)	0.90/0.08
0208 (rs3775435)	GA	Intron 4	2 (3)	157/434/272	(863)	115/439/375	(929)	0.43/0.36	7.3×10^{-6}	1.36 (1.18–1.56)	0.53/0.48
0209 (rs2737029)	TC	Intron 4	2 (1)	84/377/402	(863)	156/480/297	(933)	0.32/0.42	1.7×10^{-11}	1.60 (1.40–1.83)	0.81/0.12
0210 (rs3775442)	TC	Intron 4	2 (3)	158/438/274	(870)	114/440/378	(932)	0.43/0.36	4.2×10^{-6}	1.37 (1.19–1.58)	0.50/0.46
0211 (rs3756055)	GA	Intron 4	2 (2)	50/339/481	(870)	49/319/565	(933)	0.25/0.22	0.042	1.17 (1.00–1.37)	0.38/0.72
0212 (rs3775446)	TG	Intron 4	2 (2)	50/340/480	(870)	49/317/565	(931)	0.25/0.22	0.034	1.19 (1.01–1.38)	0.36/0.67
0213 (rs3756056)	CT	Intron 4	2 (2)	50/340/482	(872)	48/323/557	(928)	0.25/0.23	0.062	1.16 (0.99–1.34)	0.37/0.97
0214 (rs894278)	GT	Intron 4	2 (3)	156/438/275	(869)	117/441/375	(933)	0.43/0.36	1.9×10^{-5}	1.34 (1.18–1.52)	0.46/0.52
0215 (rs1812923)	CA	Intron 4	2	74/383/413	(870)	92/392/447	(931)	0.31/0.31	0.79	1.01 (0.89–1.16)	0.30/0.71
0216 (rs2298728)	AG	Intron 4	2 (3)	163/432/274	(869)	117/435/380	(932)	0.44/0.36	2.2×10^{-6}	1.38 (1.22–1.56)	0.80/0.72
0217 (rs3796667)	AT	Intron 3	2 (3)	159/430/271	(860)	114/428/383	(925)	0.44/0.36	9.2×10^{-7}	1.41 (1.23–1.61)	0.66/0.80
0218 (rs2035268)	TG	Intron 2	2 (2)	475/339/54	(868)	556/326/51	(933)	0.26/0.23	0.049	1.16 (0.99–1.37)	0.59/0.79
1023 (rs1023777)	CT	5′-flanking	2	66/318/464	(848)	86/433/411	(930)	0.27/0.33	9.3×10^{-5}	1.33 (1.15–1.55)	0.31/0.08
0220 (rs2736994)	GA		3	542/263/22	(827)	529/292/33	(854)	0.19/0.21	0.081	1.16 (0.98–1.38)	0.17/0.41
0221 (rs11097239)	CA		3	245/437/182	(864)	272/431/226	(929)	0.46/0.48	0.48	1.05 (0.92–1.19)	0.67/0.04
0222 (rs1899389)	AG		3	592/245/29	(866)	586/297/46	(929)	0.18/0.21	0.009	1.25 (1.05–1.46)	0.64/0.34
0223 (rs2289515)	AT		3	180/436/238	(854)	221/423/267	(911)	0.47/0.48	0.6	1.03 (0.90–1.18)	0.49/0.04
0224 (rs3775464)	GA		3	109/414/346	(869)	95/385/449	(929)	0.36/0.31	5.9×10^{-4}	1.28 (1.11–1.46)	0.43/0.40
0225 (rs1246270)	GA		3	372/394/84	(850)	474/372/81	(927)	0.33/0.29	0.0061	1.21 (1.05–1.40)	0.19/0.56
0226 (rs3822098)	CT		3	50/300/514	(864)	59/376/494	(929)	0.23/0.27	0.017	1.21 (1.04–1.40)	0.54/0.30

MAF, minor allele frequency. When the odds ratio (OR) is less than 1, an inverted score is indicated.
[a]Originally screened SNP.

Table 3. Haplotype association analysis using representative SNPs in block 2

Haplotypes	Representative SNP (group)						Haplotype frequency		*P*-value
	202	0070 (1)	0206 (2)	0214 (3)	0215	1023	Case	Control	
1	A	C	T	G	A	T	0.39	0.33	4.4×10^{-5}
2	T	T	T	T	A	C	0.24	0.3	5.0×10^{-6}
3	A	C	C	T	C	T	0.24	0.21	0.071
4	A	T	T	T	C	T	0.03	0.06	3.3×10^{-4}
5	T	T	T	T	C	T	0.02	0.03	0.083
6	T	T	T	G	A	T	0.01	0.02	0.62

Table 4. Association of the SNPs in group 1 of block 2

SNP	Allele	Genotype						Dominant model (MM + Mm versus mm)		Recessive model (MM versus Mm + mm)	
		Case			Control						
	M/m	MM	Mm	mm	MM	Mm	mm	*P*-value	Odds ratio (95% CI)	*P*-value	Odds ratio (95%CI)
0203	T/C	380	406	87	293	476	164	3.0×10^{-6}	1.95 (1.45–2.52)	1.0×10^{-7}	1.68 (1.41–2.07)
0204	G/A	379	399	89	289	482	159	2.7×10^{-5}	1.81 (1.36–2.38)	3.0×10^{-8}	1.72 (1.43–2.13)
0070[a]	C/T	385	394	89	295	472	165	5.7×10^{-6}	1.90 (1.44–2.53)	2.8×10^{-8}	1.71 (1.42–2.06)
0205	T/C	376	406	87	288	477	166	1.8×10^{-6}	1.98 (1.45–2.61)	6.0×10^{-8}	1.69 (1.40–2.05)
0207	T/C	382	367	91	296	482	154	5.3×10^{-4}	1.66 (1.25–2.16)	3.0×10^{-9}	1.78 (1.47–2.16)
0209	C/T	402	377	84	297	480	156	1.4×10^{-5}	1.89 (1.41–2.51)	1.5×10^{-10}	1.86 (1.55–2.27)

M and m are major allele and minor allele, respectively. CI, confidence interval.
[a]Originally screened SNP.

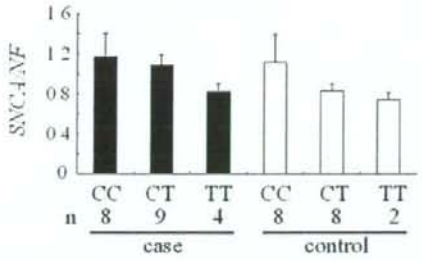

Figure 3. *In vivo* expression of *SNCA* mRNA in relation to susceptibility genotypes. SNP0070 (C/T) is used as a representative of group 1. *SNCA* expression levels in autopsied frontal cortices of cases (solid bar; 8 CC, 9 CT and 4 TT) and controls (open bar; 8 CC, 8 CT and 2 TT). Relative *SNCA* mRNA levels (normalized to *neurofilament L, NF*) are indicated. In cases, mean ± SEM of CC, CT and TT were 1.17 ± 0.23, 1.08 ± 0.11 and 0.82 ± 0.08, respectively. In controls, mean ± SEM of CC, CT and TT were 1.11 ± 0.28, 0.83 ± 0.07 and 0.75 ± 0.07, respectively.

The PD-associated alleles may positively correlate with the basal transcription level of *SNCA* and/or the induction of *SNCA* expression by certain stimulators, for example, oxidative stress.

Other possible functional effects of associated SNPs include alternative splicing, which may result in a protein isoform that aggregates more readily. The C-terminal region of SNCA is rich in acidic amino acid residues, and its truncation promotes aggregation *in vitro* (40,41). The known splice variant *SNCA112* lacks exon 5, which encodes 28 amino acids (10 of which are acidic) in frame. Thus, SNCA112 may also promote aggregation. We investigated *SNCA112* mRNA expression in frontal cortices using splice variant-specific primers, but observed little difference among the three genotypes (data not shown).

In summary, our study establishes *SNCA* as a susceptibility gene for sporadic PD. Focussed investigations of SNCA function will further enhance our understanding of how genetic factors contribute to the complex etiology of PD.

MATERIALS AND METHODS

Subjects

We recruited 882 unrelated sporadic PD patients (age, 64.9 ± 9.8; male/female ratio, 0.79) and 938 unrelated controls (age, 45.3 ± 16.3; male/female ratio, 1.10). The diagnosis of idiopathic PD was based on the presence of two or more of the cardinal features of PD (tremor, rigidity, bradykinesia and postural instability), according to the criteria for sporadic PD (42). Patients were evaluated by the certified neurologists specializing in PD. The average age of onset was 57.4 ± 10.9 years. Forty-two patients showed early onset of PD (<40 years) and 51 patients had a positive family history of PD. Patients who carried *parkin* mutations were excluded. All patients and controls were of Japanese ancestry. Informed consent was obtained from each individual, and approval for the study was obtained from the University Ethical Committees.

SNP genotyping

Genomic DNA was extracted from whole blood using FlexGene (Qiagen). SNP information was obtained from the dbSNP (http://www/ncbi.nlm.nih.gov/SNP/), JSNP (http://snp.ims.u-tokyo.ac.jp/) (43) and Celera Discovery System

(http://myscience.appliedbiosystems.com/) databases. We genotyped SNPs using the Invader assay (Third Wave Technologies), TaqMan (Applied Biosystems) or direct sequencing using an ABI3730 capillary sequencer (Applied Biosystems). Rep1 genotyping and allele designations followed those described previously (35). The Rep1 region was amplified using FAM5′-CCTGGCATATTTGATTGCAA-3′ and 5′-GACTGGCCCAAGATTAACCA-3′ as primers and analyzed using ABI3730 capillary sequencer.

Statistical analysis

SNPAlyze software (DYNACOM, Japan) was used for the case–control study (χ^2-test), calculation of odds ratio and its 95% CI (Bootstrap method), haplotype analysis (Expectation–Maximization algorithm) and pairwise LD analysis (Lewontin's coefficient D' and standardized coefficient r).

Real-time RT–PCR

Autopsied frontal cortices were obtained from the Brain Bank for Aging Research (Tokyo Metropolitan Geriatric Hospital/Tokyo Metropolitan Institute of Gerontology) and from the Department of Neurology, Juntendo University School of Medicine. The samples contained 21 cases [age, 82.6 ± 7.1 (SD) years; 11 males and 10 females] with Lewy body pathology defined by the third Consensus Guideline for Dementia with Lewy Bodies (44), comprising PD with and without dementia and dementia with Lewy bodies, and 18 control subjects (age, 81.2 ± 5.2; 12 males and six females) without parkinsonism or dementia and without neurodegenerative pathological changes. Total RNA was extracted from tissues using RNeasy (Qiagen), and cDNA was prepared using Superscript reverse transcriptase (Invitrogen). Real-time RT–PCR was carried out on ABI PRISM 7900 sequence detection system (Applied Biosystems) using SYBR Premix Ex Taq (TAKARA, Japan). First-strand cDNA was amplified using primers specific for *SNCA* (forward: 5′-GCAGAAGCA GCAGGAAAGAC-3′; reverse: 5′-CTGGGCTACTGCTGTC ACAC-3′; product size: 159 bp) and *NF* (*neurofilament L*, forward: 5′-AGAACGCTGAGGAATGGTTC-3′; reverse: 5′-CTGGTGAAACTGAGTCGGGT-3′; product size: 391 bp). A single band of the expected size was amplified from cDNA samples, but not from RNA samples. For quantification, we used a relative standard curve method. Standard curves of *SNCA* and *NF* were generated from the amplification of diluted series of cDNA from cortices. *SNCA* expression levels were normalized to those of *NF*. One of the experimental samples was used as the calibrator. Each of the normalized *SNCA* values was divided by the calibrator normalized *SNCA* value to generate the relative expression levels. The values were determined in triplicate. Reproducibility of the results was confirmed by repeating cDNA synthesis and real-time PCR twice for seven samples, and similar results were obtained.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES

1. Rascol, O., Payoux, P., Ory, F., Ferreira, J.J., Brefel-Courbon, C. and Montastruc, J.-L. (2003) Limitations of current Parkinson's disease therapy. *Ann. Neurol.*, **53** (Suppl. 3), S3–S12.
2. Warner, T.T. and Schapira, A.H. (2003) Genetic and environmental factors in the cause of Parkinson's disease. *Ann. Neurol.*, **53** (Suppl. 3), S16–S23.
3. Elbaz, A., Grigoletto, F., Baldereschi, M., Breteler, M.M., Manubens-Bertran, J.M., Lopez-Pousa, S., Dartigues, J.F., Alperovitch, A., Tzourio, C., Rocca, W.A. *et al.* (1999) Familial aggregation of Parkinson's disease: a population-based case–control study in Europe. *Neurology*, **52**, 1876–1882.
4. Sveinbjörnsdóttir, S., Hicks, A.A., Jónsson, T., Pétursson, H., Gugmundsson, G., Frigge, M.L., Kong, A., Gulcher, J.R. and Stefánsson, K. (2000) Familial aggregation of Parkinson's disease in Iceland. *N. Engl. J. Med.*, **343**, 1765–1770.
5. Piccini, P., Burn, D.J., Ceravolo, R., Maraganore, D. and Brooks, D.J. (1999) The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. *Ann. Neurol.*, **45**, 577–582.
6. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. *et al.* (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
7. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
8. Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T. *et al.* (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 451–452.
9. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G. *et al.* (2004) Hereditary early-onset Parkinson's disease caused by mutations in *PINK1*. *Science*, **304**, 1158–1160.
10. Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C.J., Squitieri, F., Ibanez, P., Joosse, M. *et al.* (2003) Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. *Science*, **299**, 256–259.
11. Paisán-Ruiz, C., Jain, S., Evans, E.W., Gilks, W.P., Simón, J., van der Brug, M., Lopez de Munain, A., Aparicio, S., Gil, A.M., Khan, N. *et al.* (2004) Cloning of the gene containing mutations that cause *PARK8*-linked Parkinson's disease. *Neuron*, **44**, 595–600.
12. Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R.J., Calne, D.B. *et al.* (2004) Mutations in *LRRK2* cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron*, **44**, 601–607.

13. Le, W.-D., Xu, P., Jankovic, J., Jiang, H., Appel, S.H., Smith, R.G. and Vassilatis, D.K. (2003) Mutations in *NR4A2* associated with familial Parkinson disease. *Nat. Genet.*, **33**, 85–89.
14. DeStefano, A.L., Golbe, L.I., Mark, M.H., Lazzarini, A.M., Maher, N.E., Saint-Hilaire, M., Feldman, R.G., Guttman, M., Watts, R.L., Suchowersky, O. *et al.* (2001) Genome-wide scan for Parkinson's disease: the *Gene*PD Study. *Neurology*, **57**, 1124–1126.
15. Hicks, A.A., Pétursson, H., Jónsson, T., Stefánsson, H., Jóhannsdóttir, H.S., Sainz, J., Frigge, M.L., Kong, A., Gulcher, J.R., Stefánsson, K. *et al.* (2002) A susceptibility gene for late-onset idiopathic Parkinson's disease. *Ann. Neurol.*, **52**, 549–555.
16. Pankratz, N., Nichols, W.C., Uniacke, S.K., Halter, C., Rudolph, A., Shults, C., Conneally, P.M., Foroud, T. and the Parkinson Study Group (2002) Genome screen to identify susceptibility genes for Parkinson disease in a sample without *parkin* mutations. *Am. J. Hum. Genet.*, **71**, 124–135.
17. Scott, W.K., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Lyons, K., Pahwa, R., Stern, M.B., Colcher, A., Hiner, B.C. *et al.* (2001) Complete genomic screen in Parkinson disease: evidence for multiple genes. *JAMA*, **286**, 2239–2244.
18. Martin, E.R., Scott, W.K., Nance, M.A., Watts, R.L., Hubble, J.P., Koller, W.C., Lyons, K., Pahwa, R., Stern, M.B., Colcher, A. *et al.* (2001) Association of single-nucleotide polymorphisms of the tau gene with late-onset Parkinson disease. *JAMA*, **286**, 2245–2250.
19. van der Walt, J.M., Noureddine, M.A., Kittappa, R., Hauser, M.A., Scott, W.K., McKay, R., Zhang, F., Stajich, J.M., Fujiwara, K., Scott, B.L. *et al.* (2004) Fibroblast growth factor 20 polymorphisms and haplotypes strongly influence risk of Parkinson disease. *Am. J. Hum. Genet.*, **74**, 1121–1127.
20. Spillantini, M.G., Schmidt, M.L., Lee, V.M.-Y., Trojanowski, J.Q., Jakes, R. and Goedert, M. (1997) α-synuclein in Lewy bodies. *Nature*, **388**, 839–840.
21. Goedert, M. (2001) Alpha-synuclein and neurodegenerative diseases. *Nat. Rev. Neurosci.*, **2**, 492–501.
22. Schildkraut, J.M. (1998) Examining complex genetic interactions. In Haines, J.L. and Pericak-Vance, M.A. (eds), *Approaches to Gene Mapping in Complex Human Diseases*. Wiley-Liss, NY, pp. 379–410.
23. Mueller, J.C., Fuchs, J., Hofer, A., Zimprich, A., Lichtner, P., Illig, T., Berg, D., Wüllner, U., Meitinger, T. and Gasser, T. (2005) Multiple regions of α-synuclein are associated with Parkinson's disease. *Ann. Neurol.*, **57**, 535–541.
24. Pals, P., Lincoln, S., Manning, J., Heckman, M., Skipper, L., Hulihan, M., Van den Broeck, M., De Pooter, T., Cras, P., Crook, J. *et al.* (2004) α-Synuclein promoter confers susceptibility to Parkinson's disease. *Ann. Neurol.*, **56**, 591–595.
25. Maroteaux, L., Campanelli, J.T. and Scheller, R.H. (1988) Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J. Neurosci.*, **8**, 2804–2815.
26. Eriksen, J.L., Dawson, T.M., Dickson, D.W. and Petrucelli, L. (2003) Caught in the act: α-synuclein is the culprit in Parkinson's disease. *Neuron*, **40**, 453–456.
27. Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J.T., Schöls, L. and Riess, O. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet.*, **18**, 106–108.
28. Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B. *et al.* (2004) The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.*, **55**, 164–173.
29. Nagar, S., Juyal, R.C., Chaudhary, S., Behari, M., Gupta, M., Rao, S.N. and Thelma, B.K. (2001) Mutations in the α-synuclein gene in Parkinson's disease among Indians. *Acta Neurol. Scand.*, **103**, 120–122.
30. Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R. *et al.* (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science*, **302**, 841.
31. Miller, D.W., Hague, S.M., Clarimon, J., Baptista, M., Gwinn-Hardy, K., Cookson, M.R. and Singleton, A.B. (2004) α-synuclein in blood and brain from familial Parkinson disease with *SNCA* locus triplication. *Neurology*, **62**, 1835–1838.
32. Chartier-Harlin, M.-C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M. *et al.* (2004) α-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet*, **364**, 1167–1169.
33. Ibáñez, P., Bonnet, A.-M., Débarges, B., Lohmann, E., Tison, F., Pollak, P., Agid, Y., Dürr, A., Brice, A. and French Parkinson's Disease Genetics Study Group (2004) Causal relation between α-synuclein gene duplication and familial Parkinson's disease. *Lancet*, **364**, 1169–1171.
34. Xia, Y., Rohan de Silva, H.A., Rosi, B.L., Yamaoka, L.H., Rimmler, J.B., Pericak-Vance, M.A., Roses, A.D., Chen, X., Masliah, E., DeTeresa, R. *et al.* (1996) Genetic studies in Alzheimer's disease with an NACP/α-synuclein polymorphism. *Ann. Neurol.*, **40**, 207–215.
35. Farrer, M., Maraganore, D.M., Lockhart, P., Singleton, A., Lesnick, T.G., de Andrade, M., West, A., de Silva, R., Hardy, J. and Hernandez, D. (2001) α-synuclein gene haplotypes are associated with Parkinson's disease. *Hum. Mol. Genet.*, **10**, 1847–1851.
36. Parsian, A., Racette, B., Zhang, Z.H., Chakraverty, S., Rundle, M., Goate, A. and Perlmutter, J.S. (1998) Mutation, sequence analysis, and association studies of α-synuclein in Parkinson's disease. *Neurology*, **51**, 1757–1759.
37. Tan, E.-K., Tan, C., Shen, H., Chai, A., Lum, S.-Y., Teoh, M.-L., Yih, Y., Wong, M.-C. and Zhao, Y. (2003) Alpha synuclein promoter and risk of Parkinson's disease: microsatellite and allelic size variability. *Neurosci. Lett.*, **336**, 70–72.
38. Jobling, M.A., Hurles, M. and Tyler-Smith, C. (2004) *Human Evolutionary Genetics*. Garland Science, NY, pp. 45–86.
39. Altshuler, D., Brooks, L.D., Chakravarti, A., Collins, F.S., Daly, M.J., Donnelly, P. and the International HapMap Consortium (2005) A haplotype map of the human genome. *Nature*, **437**, 1299–1320.
40. Murray, I.V.J., Giasson, B.I., Quinn, S.M., Koppaka, V., Axelsen, P.H., Ischiropoulos, H., Trojanowski, J.Q. and Lee, V.M.-Y. (2003) Role of α-synuclein carboxy-terminus on fibril formation *in vitro*. *Biochemistry*, **42**, 8530–8540.
41. Serpell, L.C., Berriman, J., Jakes, R., Goedert, M. and Crowther, R.A. (2000) Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-β conformation. *Proc. Natl Acad. Sci. USA*, **97**, 4897–4902.
42. Bower, J.H., Maraganore, D.M., McDonnell, S.K. and Rocca, W.A. (1999) Incidence and distribution of Parkinsonism in Olmsted County, Minnesota, 1976–1990. *Neurology*, **52**, 1214–1220.
43. Haga, H., Yamada, R., Ohnishi, Y., Nakamura, Y. and Tanaka, T. (2002) Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190 562 genetic variations in the human genome. *J. Hum. Genet.*, **47**, 605–610.
44. McKeith, I.G., Dickson, D.W., Lowe, J., Emre, M., O'Brien, J.T., Feldman, H., Cummings, J., Duda, J.E., Lippa, C., Perry, E.K. *et al.* (2005) Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. *Neurology*, **65**, 1863–1872.

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Beta-site APP cleaving enzyme 1 (BACE1) is increased in remaining neurons in Alzheimer's disease brains

Hirotsugu Harada [a], Akira Tamaoka [a,*], Kazuhiro Ishii [a], Shin'ichi Shoji [a], Satoshi Kametaka [b], Fuyuki Kametani [c], Yuko Saito [d], Shigeo Murayama [d]

[a] *Department of Neurology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[b] *Osaka University, Osaka, Japan*
[c] *Tokyo Institute of Psychiatry, Tokyo, Japan*
[d] *Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*

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Abstract

Alzheimer's disease (AD) is characterized by the extensive deposition of amyloid β protein (Aβ) in the brain cortex. Aβ is produced from β-amyloid precursor protein (APP) by β-secretase and γ-secretase. β-Secretase has been identified as beta-site APP cleaving enzyme1 (BACE1). We produced rabbit polyclonal antibodies against the amino and the carboxyl terminals of BACE1. Using these antibodies, BACE1 was characterized in temporal lobe cortices by Western blotting and immunohistochemistry.

Immunohistochemical studies employing anti-GFAP and anti-MAP2 antibodies as well as anti-BACE1 antibodies showed that BACE1 was expressed exclusively in neurons but not in glial cells.

Brain samples were directly extracted by 0.5% SDS and analyzed by Western blotting and densitometer. Although the mean level of BACE1/mg protein in AD brains was not increased, the ratio of BACE1 to MAP2 or to NSE was significantly increased compared with that in control brains.

Taken together, these findings suggest that those neurons that survive in AD brains might generate more BACE1 than normal neurons in control brains, indicating that increased BACE1 activity could be one of the causes of AD. This could justify the development of anti-BACE1 drugs for AD treatment.

Keywords: Alzheimer's disease; Aβ; BACE1

1. Introduction

Alzheimer's disease (AD) is a major form of senile dementia. The appearance of senile plaques with β-amyloid protein (Aβ) as the main component precedes the various pathological changes observed in AD brains (Glenner and Wong, 1984). Aβ is produced by cleavage of Aβ precursor protein (APP) at the amino terminal end by β-secretase and at the carboxyl terminal end by γ-secretase (Hardy and Allsop, 1991). Part of the Aβ released outside the cell is degraded and removed by degradative enzymes; however, increased Aβ production, reduced activity of the degradative enzymes, or reduced activity of the mechanism for removal of Aβ causes the formation of senile plaques through the aggregation and deposition of Aβ (Selkoe, 1999). Furthermore, aggregated Aβ acts against neuronal cells and causes neuronal cell death. It is also said that Aβ activates the phosphorylation of tau protein, causing neurofibrillary degeneration (Rapoport et al., 2002; King, 2005). Therefore, it is considered that inhibition of Aβ production before its deposition, enhancement of Aβ degradation, or clearance of deposited Aβ would be effective in the prevention and treatment of AD. Two phenotypes of BACE have been observed: BACE1 and BACE2. BACE1 is considered to be the major β-secretase because: (1) it exists mainly in the brain, whereas BACE2 is widely distributed throughout the body; (2) only small amounts of BACE2 mRNA have been observed in the brain (Vassar et al., 1999; Bennett et al., 2000; Vassar, 2004). Furthermore, although Aβ production is

* Corresponding author. Fax: +81 29 853 3224.
E-mail addresses: haradah@nona.dti.ne.jp (H. Harada), atamaoka@md.tsukuba.ac.jp (A. Tamaoka).

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increased when human BACE1 is overexpressed in transgenic mice that express a Swedish variant of APP, it is not observed in BACE1 knockout mice (Bodendorf et al., 2002; Luo et al., 2001). These findings suggest that BACE1 inhibitors would suppress the production of Aβ and might be useful as new therapies for AD. To study the localization of BACE1 in AD brains, we prepared anti-human BACE1 antibodies and investigated their reactivity and specificity. We also compared the quantities of BACE1 between normal and AD brains. In addition, we analyzed the relationship between the amount of BACE1 and Aβ species, as measured by enzyme-linked immunosorbent assay (ELISA).

2. Methods

2.1. Case selection

We used frozen temporal lobe cortices (Brodmann area 21) preserved in this hospital and at the Tokyo Metropolitan Institute of Gerontology, including 28 AD cases and 25 normal controls. All AD cases had been diagnosed pathologically according to the criteria of the Reagan Institute Working Group/National Institute on Aging (stages 5 and 6 according to Braak and Braak) (Braak and Braak, 1991; The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997), having been clinically and pathologically differentiated from dementia caused by other diseases. All control subjects had been examined clinically and pathologically. Their causes of death were cerebrovascular infarction or non-neurological diseases. The temporal, frontal, occipital, and parietal lobes from the control brains had been confirmed as pathologically normal for age. No significant differences between AD and control cases were evident concerning distributions of age, gender, or postmortem interval (range, 3–12 h).

2.2. Cultured cells as positive controls expressing human BACE1

pcDNA3-hBACE1 was prepared by cloning full-length BACE1 from the human brain library and then inserting pcDNA3. Next, the construct was transiently forced to express itself in HeLa cells and treated in a lysis buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% NP40, 1% Triton-X100, 2 mM EDTA, protease inhibitor cocktail [Roche, Penzberg, Germany], 1 mM PMSF) on ice for 30 min. The resultant solution was centrifuged at 100 000 × *g* for 15 min to obtain the lysate, the supernatant of this process.

2.3. Preparation of antibodies

Rabbits were immunized with synthetic peptides of the amino terminal (45–55, ETDEEPEEPGR) and the carboxyl terminal (485–501, CLRQQHDDFADDISLLK) of BACE1 to prepare polyclonal antibodies, referred to here as anti-BACE1-N antibody and anti-BACE1-C antibody, respectively.

2.4. Preparation of brain samples and Western blotting

To determine the solubility of BACE1, brain samples were homogenized in a buffer (described below) with three times the volume of the sample, and the whole homogenate was then centrifuged at 100 000 × *g* for 20 min to separate the supernatant. Consecutive extraction was performed with TSE (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA), 0.1% Triton-X100/TSE, and 0.5% SDS/TSE, which were added with the inhibitors of proteolytic enzymes (Roche).

The samples of each supernatant and the final pellets were heat-blocked for 10 min in a loading buffer (125 mM Tris–HCl, 20% glycerol, 10% 2-mercaptethanol, 4% SDS, 0.02% bromophenol blue, pH 6.8) and then subjected to electrophoresis on a 10–20% Tris–glycine sodium dodecyl sulfate–polyacrylamide gel (Real Gel Plate, Biocraft, Tokyo, Japan). The samples were then electrically transferred to a transfer membrane (Millipore, Billerico, MA) and blocked for 1 h in phosphate buffered saline (PBS) containing 10% skim milk and 0.1% Tween 20. Anti-BACE1-N antibody (1:2000) and anti-BACE1-C antibody (1:5000) were incubated at 4 °C overnight in a PBS buffer containing 5% bovine albumin. The membrane was rinsed with PBS buffer containing 0.1% Tween 20, incubated with HRP-labeled anti-rabbit IgG (1:5000, Dako-Cytomation, Glostrup, Denmark) for 3 h, and then stained with the detection reagents (Amersham, Buckinghamshire, UK).

Subsequently, direct extraction of a control brain was performed with 0.5% SDS/TSE, which could dissolve more proteins than the other two buffers (TSE or 0.1% Triton-X100/TS), and Western blotting was performed by using 1132-N (1:1000) and 1134-C (1:1000) as authentic BACE1 antibodies against amino acids 45–56 and 487–501, respectively, in addition to anti-BACE1-N antibody (1:2000) and anti-BACE1-C antibody (1:5000).

To study post-translational glycosidation of BACE1, control brain extracts with 0.5% SDS/TSE were incubated at 37 °C for 14 h using an N-Glycosidase F Deglycosylation Kit (Roche), and Western blotting was performed by using anti-BACE1-N antibody and anti-BACE1-C antibody.

To compare total quantities of BACE1, the brains of the 25 normal controls and 28 AD patients were extracted with 0.5% SDS/TSE. After protein amounts were measured by the bicinchoninic acid method (PIERCE, Rockford, IL), concentrations of protein in each sample were adjusted to be equal. Anti-BACE1-C antibody (1:5000), anti-microtubule-associated protein 2 (MAP2) antibody (Sigma, St. Louis, MO), and anti-neuron specific enolase (NSE) antibody (IBL, Gunma, Japan) were used as the primary antibodies, and HRP-labeled anti rabbit antibody was used as the secondary antibody for Western blotting. Measurement and comparison were performed with a densitometer (GS-710, Quantity One, Bio-Rad, Richmond, CA).

2.5. Double immunostaining

Double immunostaining was performed on 6-μm-thick paraffin sections of the temporal lobes from AD brains. After deparaffinization, blocking was performed using PBS containing 5% goat serum and 0.1% Tween 20, then staining was performed with anti-BACE1-C antibody (1:500) as the primary antibody and anti-rabbit IgG (H + L) Fluor 488 (1:400, Molecular Probes Europe BV, Leiden, The Netherlands) as the secondary antibody. Double staining was then performed using anti-MAP2 antibody and anti-GFAP antibody (Progen, Heidelberg, Germany) as the first antibodies and anti-mouse IgG (H + L) Fluor 633 (Molecular Probes Europe BV) as the second antibody. Confocal images were obtained under a confocal laser microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany).

2.6. Quantification of Aβ by sandwich-type enzyme-linked immunosorbent assay (ELISA)

Species of Aβ were measured using the same parts of the temporal lobe cortices that had been used to measure BACE1. Wet tissue (0.5 g) from the cortex of each patient was finely minced, homogenized in 2 ml of 99% formic acid, and centrifuged at 100 000 × *g* for 60 min at 4 °C. The supernatant was neutralized with 1N NaOH, diluted, and subjected to ELISA for Aβ quantitation. To immunochemically identify and quantify different species of Aβ in the cortex, we used a sandwich ELISA with BA27 and BC05, which respectively are HRP-labeled antibodies capable of distinguishing the differing COOH-termini of Aβ 1–40 and 1–42. The sandwich ELISA for Aβ was carried out as described previously (Tamaoka et al., 1995, 1997). Briefly, 100 μl of a standard peptide to establish antibody specificity or a prepared patient sample was placed in a microtiter plate wells previously coated with BNT77, a monoclonal antibody against the NH_2-terminal sequence of Aβ (1–16). Samples were allowed to react at 4 °C for 24 h. After washing with PBS, plates were incubated at 4 °C for 24 h with wells containing HRP-labeled BA27 or BC05, the secondary antibodies for differential measurement of Aβ 40 and Aβ 42, respectively. HRP activities bound to antibodies reacting with samples were assayed with a microtiter plate reader after color development using the TMB microwell peroxidase system (KPL, Gaithersburg, MD).

3. Results

3.1. A 70-kDa protein is detected in the cerebral cortex by anti-BACE1-C antibody and anti-BACE1-N antibody

Supernatants of consecutive extractions from the temporal cortex, final pellets, and positive controls were analyzed on immunoblots with anti-BACE1-C antibody and anti-BACE1-N antibody. Fractions consecutively extracted with TSE, 1% Triton-X100/TSE, and 0.5% SDS/TSE revealed a band migrating at ~70 kDa in all supernatants; however, no band was observed at the same position in the final pellet (Fig. 1a). Since each consecutively extracted fraction contained ~70 kDa protein, Western blotting was performed thereafter on prepared samples obtained by direct extraction of brains with 0.5% SDS/TSE. All these antibodies detected a band migrating at 70 kDa in such fractions (Fig. 1b).

We considered that we had identified mature BACE1, because we observed a band at 70 kDa with both the anti-BACE1-N antibody and the anti-BACE1-C antibody, and it comigrated with a protein with the molecular weight of BACE1, as already reported (Haniu et al., 2000). Deglycosylation of the positive controls with N-glycosidase F resulted in disappearance of the band at 70 kDa and appearance of a band at 50 kDa. Thus, the full-length mature BACE1 was confirmed as the glycosylated form of BACE1 (Fig. 2). Western blotting of control brains with anti-BACE1-C antibody and anti-BACE1-N antibody revealed a band at the same position as the deglycosylated form of BACE1 after treatment with N-glycosidase F.

3.2. BACE1 is expressed mainly in neurons

To determine whether expression of BACE1 in the AD brain occurred mainly in neurons or in astrocytes accompanying the gliosis, we performed double immunostaining of the temporal lobes of AD and normal brains. Stainings of the cortices of the temporal lobe and the hippocampus against anti-BACE1-C antibody were consistent with those for anti-MAP2 antibody. Astrocytes stained for anti-GFAP antibody were not labeled by anti-BACE1-C antibody (Fig. 3). These findings led us to consider that BACE1 was expressed mainly in the neurons, not in glial cells.

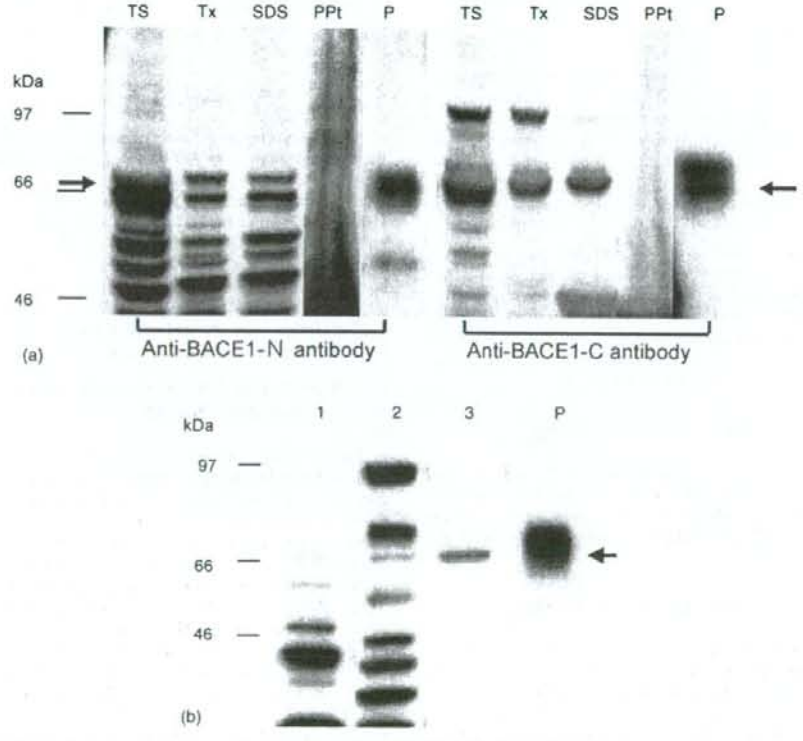

Fig. 1. (a) Immunoblots of sequential extractions of control brain stained with anti-BACE1-N antibody (left) and anti-BACE1-C antibody (right). The acrylamide gel gradient was 10–20%. Lanes: TS, supernatant directly extracted by TSE; Tx, supernatant sequentially extracted by 1% Triton-X100/TSE; SDS, supernatant sequentially extracted by 0.5% SDS/TSE; PPt, final pellet; P, positive control. All brain fractions extracted sequentially by TS, Tx, and SDS were revealed to contain 70 kDa protein (arrow), but PPt did not. (b) Immunoblots of supernatant directly extracted by 0.5% SDS from control brain stained with various anti-BACE1 antibodies. The acrylamide gel gradient was 10–20%. Lanes: 1, 1132-N (1:1000); 2, 1134-C (1:1000); 3, anti-BACE1-N antibody (1:2000); 4, anti-BACE1-C antibody (1:5000); P, positive control stained with anti-BACE1-C antibody. All antibodies detected 70-kDa protein (arrow).

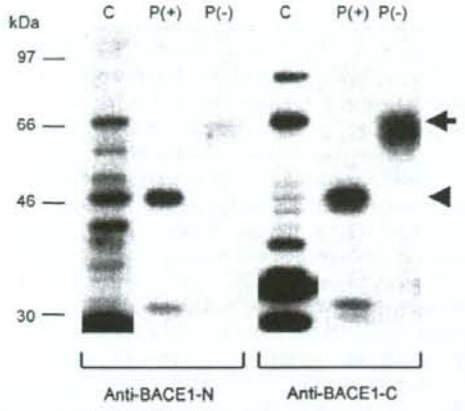

Fig. 2. Immunoblots of recombinant BACE1 (positive control) digested by N-glycosidase F. Lanes; C, supernatant directly extracted by 0.5% SDS from a control brain (untreated); P(+), positive control digested by N-glycosidase F; P(−), untreated positive control. Digestion of positive control by N-glycosidase F altered the molecular weight from 70 (arrow) to 50 kDa (arrowhead), which was consistent with the molecular weight of BACE1 calculated from its amino acid residues. The 70- and 50-kDa bands were also detected in immunoblots of a control brain with anti-BACE1-N antibody (left) and anti-BACE1-C antibody (right).

3.3. BACE1 expression is increased in remaining neurons in AD brains

Because double immunostaining suggested that BACE1 was expressed mainly in neurons, we performed Western blotting with anti-BACE1-C antibody as well as with anti-MAP2 antibody and anti-NSE antibody, measuring the amounts of these proteins (Fig. 4). There were no significant differences in the total amounts of BACE1 between AD and control brains. However, significant decreases in the levels of MAP2 and NSE were observed in the AD group compared with the normal group: the amount of MAP2 in the AD group as a proportion of that in the normal group was 0.29 ($p < 0.05$), and that of NSE was 0.31 ($p < 0.001$). These results were assumed to reflect neuronal loss in the AD brains. The ratios of BACE1 to MAP2 (BACE1/MAP2) and NSE (BACE1/NSE) were 3.0 and 4.6 (both $p < 0.05$), respectively, in the AD group (Fig. 5). We considered that these ratios reflected the relative amounts of BACE1 per neuron. There was a tendency towards an increase in BACE1 concentration with age in both groups, although this trend was not significant. This tendency was greater in AD brains than in the controls (data not shown).

Sandwich ELISA showed that the levels of both Aβ 40 and Aβ 42 were increased in the AD group, as shown previously

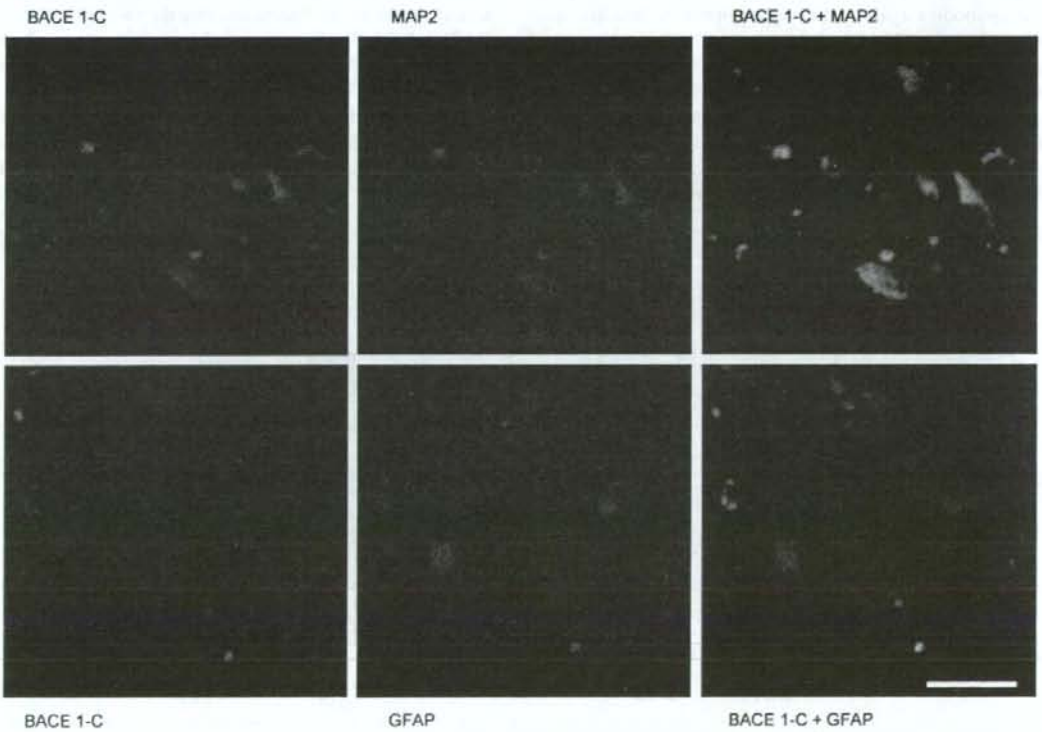

Fig. 3. Immunohistochemical studies of temporal cortex from AD brain, employing anti-MAP2 antibody (upper row, red) and anti-GFAP antibody (lower row, red) as well as anti-BACE1-C antibody (green). BACE1 was expressed exclusively in neurons but not in astrocytes (bar = 40 μm).

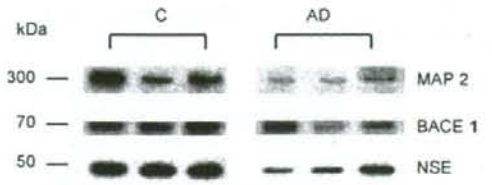

Fig. 4. Western blottings using anti-BACE1-C antibody, anti-MAP2, and anti-NSE in AD and control brains. The samples were extracted with 0.5% SDS/TSE and the concentration of protein in each sample was adjusted to the same level.

(Tamaoka et al., 1995); however, we observed no correlation between the concentrations of BACE1 and Aβ species.

4. Discussion

BACE1 is first synthesized as a transmembrane protein with 501 amino acids. The 1–21 amino acid part of the protein represents a signal peptide and the adjacent 22–45 part represents a proprotein domain. The remaining 46–501 part is considered to undergo maturation through endoplasmic reticulum to become the final 70 kDa mature BACE1 (Vassar et al., 1999; Haniu et al., 2000; Vassar, 2004). Several findings support the identity of the 70-kDa protein recognized by our antibodies as BACE1. (i) In addition to our two antibodies (anti-BACE1-N and anti-BACE1-C), 1132-N and 1134-C, polyclonal antibodies against carboxyl-terminal of BACE1, also detected the same 70-kDa protein band. (ii) Few numbers of extrabands were observed in the Western blottings using anti-BACE1-C antibody in AD and control brains. (iii) Both our novel antibodies against BACE1 immunostained recombinant BACE1 as the band migrating at 70 kDa and deglycosylated recombinant BACE1 at 50 kDa, which is consistent with the molecular weight of BACE1 calculated from its amino acid composition. In the light of the molecular weight of BACE1 calculated by its amino acid composition, with 46–501 amino acid residues, we considered the 50 kDa band that appeared after treatment with N-glycosidase F in the positive controls to be deglycosylated BACE1. The same deglycosylated forms of BACE1 could be identified in normal brains. These findings implied that unmodified BACE1 was present in human brains.

Expression of BACE1 was confirmed by double immunofluorescence staining to occur exclusively in the neurons, as reported previously (Seubert et al., 1993; Zhao et al., 1996). Therefore, we measured the amount of BACE1 protein as well as the amounts of MAP2 and NSE, as neuron-specific proteins, and then examined the ratios of BACE1/MAP2 and BACE1/NSE, which we considered to reflect the level of BACE1 expression per neuron.

We concluded that BACE1 expression per neuron was increased, although the total amount of BACE1 expression in the cortices of the temporal lobe was not increased. This observation implied that Aβ production per neuron might increase before cell death. Fukumoto et al. reported that the total amount of BACE1 protein in the temporal lobes of AD brains tended to increase, as measured by sandwich ELISA using mouse monoclonal anti BACE1 carboxyl terminal antibody and rabbit polyclonal anti BACE1 amino terminal antibody. From the amount of synaptophysin, as measured by ELISA, they also reported that BACE1 expression per neuron was increased in AD brains, because the value of the amount of BACE1 divided by the amount of synaptophysin was increased (Fukumoto et al., 2002). There results are fairly consistent with ours.

Yang et al. reported elevated BACE1 expression and activity in sporadic AD. By using Western blotting analysis they showed that BACE1 levels were significantly higher in the temporal cortex in AD than in non-demented controls. The

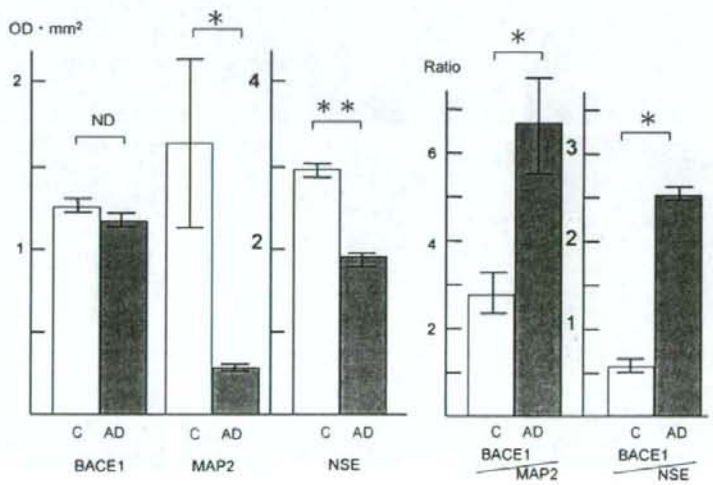

Fig. 5. Protein levels of BACE1, MAP2, and NSE in AD and control (C) brains. The amount of BACE1/mg protein in AD brains was slightly decreased in comparison with that in control brains. However, in AD, the ratios of BACE1 to MAP2 (BACE1/MAP2) and BACE1 to NSE (BACE1/NSE) were significantly increased compared with those in control brains (BACE1/MAP2 and BACE1/NSE $p < 0.05$) ($^{*}p < 0.05$; $^{**}p < 0.0001$).

differences in results between their study and ours could be due to several factors. (i) They used rapidly autopsied brains of AD patients (<3 h), although they did not comment on the postmortem intervals of the non-demented controls. (ii) They normalized BACE1 expression against β-actin. (iii) The AD brain samples that they used might have contained quite large quantities of neurons, as suggested by their levels of MAP-2 and NSE expression. Further studies using brains examined after different postmortem intervals and samples from different brain lesions are needed before we can draw definite conclusions on BACE1 expression (Yang et al., 2003).

Production of Aβ is not seen in BACE1 knockout mice, and no abnormalities are observed in these mice (Luo et al., 2001; Roberds et al., 2001). On the other hand, γ-secretase, which is the cleavage enzyme at the carboxyl terminal side of APP, is also involved in the cleavage of other proteins such as Notch1. Notch1 is an important substance involved in the differentiation and transportation of cells during the embryonic period. It is also known that Notch1 is involved in the differentiation of immune cells in the body, and that inhibition of its cleavage activity causes immune abnormalities (Petit et al., 2001). Therefore, we consider that reduction in the amount of BACE1 is less likely to cause side effects than targeting of γ-secretase.

Several lines of evidence have led to postulation that Aβ accumulation comes from enhancement of its production, promotion of its aggregation, or inhibition of its degradation or excretion (Selkoe, 1999; Hardy and Selkoe, 2002; Iwata et al., 2001). Our results revealed no correlation between the amount of BACE1 protein and the accumulated amount of Aβ species, suggesting that several other factors could cause abnormal accumulation of Aβ in the AD brain. However, BACE1 inhibitors could still provide a new therapy for AD, possibly through inhibition of additional production of Aβ. This is supported by the fact that our study revealed increased production of BACE1 by the surviving neurons in AD brains, and by a previous report that Aβ production is not detected in BACE1 knockout mice.

Acknowledgments

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References

Bennett, B.D., Babu-Khan, S., Loeloff, R., Louis, J.C., Curran, E., Citron, M., Vassar, R., 2000. Expression analysis of BACE2 in brain and peripheral tissues. J. Biol. Chem. 275, 20647–20651.

Bodendorf, U., Danner, S., Fischer, F., Stefani, M., Sturchler-Pierrat, C., Wiederhold, K.H., Staufenbiel, M., Paganetti, P., 2002. Expression of human beta-secretase in the mouse brain increases the steady-state level of beta-amyloid. J. Neurochem. 80, 799–806.

Braak, H., Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. (Berlin) 82, 239–259.

Fukumoto, H., Cheung, B.S., Hyman, B.T., Iraozarry, M.C., 2002. β-Secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch. Neurol. 59, 1381–1389.

Glenner, G.G., Wong, C.W., 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Biophys. Res. Commun. 120, 885–890.

Haniu, M., Denis, P., Young, Y., Mendiaz, E.A., Fuller, J., Hui, J.O., Bennett, B.D., Kahn, S., Ross, S., Burgess, T., Katta, V., Rogers, G., Vassar, R., Citron, M., 2000. Characterization of Alzheimer's beta-secretase protein BACE. A pepsin family member with unusual properties. J. Biol. Chem. 275, 21099–21106.

Hardy, J., Allsop, D., 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Phamac. 12, 383–388.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353–356.

Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N.P., Gerard, C., Hama, E., Lee, H.J., Saido, T.C., 2001. Metabolic regulation of brain Abeta by neprilysin. Science 292, 1550–1552.

King, M.E., 2005. Can tau filaments be both physiologically beneficial and toxic? Biochim. Biophys. Acta 1739, 260–267.

Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J.C., Yan, Q., Richards, W.G., Citron, M., Vassar, R., 2001. Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nat. Neurosci. 4, H231–H232.

Petit, A., Bihel, F., Alves da Costa, C., Pourquie, O., Checler, F., Kraus, J.L., 2001. New protease inhibitors prevent gamma-secretase-mediated production of Abeta 40/42 without affecting Notch cleavage. Nat. Cell Biol. 3, 507–511.

Rapoport, M., Dawson, H.N., Binder, L.I., Vitek, M.P., Ferreira, A., 2002. Tau is essential to beta-amyloid-induced neurotoxicity. Proc. Natl. Acad. Sci. U.S.A. 99, 6364–6369.

Roberds, S.L., Anderson, J., Basi, G., Bienkowski, M.J., Branstetter, D.G., Chen, K.S., Freedman, S.B., Frigon, N.L., Games, D., Hu, K., Johnson-Wood, K., Kappenman, K.E., Kawabe, T.T., Kola, I., Kuehn, R., Lee, M., Liu, W., Motter, R., Nichols, N.F., Power, M., Robertson, D.W., Schenk, D., Schoor, M., Shopp, G.M., Shuck, M.E., Sinha, S., Svensson, K.A., Tatsuno, G., Tintrup, H., Wijsman, J., Wright, S., McConlogue, L., 2001. BACE knockout mice are healthy despite lacking the primary beta-secretase activity in brain: implications for Alzheimer's disease therapeutics. Hum. Mol. Genet. 10, 1317–1324.

Selkoe, D.J., 1999. Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399, A23–A31.

Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., et al., 1993. Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. Nature 361, 260–263.

Tamaoka, A., Sawamura, N., Fukushima, T., Shoji, S., Matsubara, E., Shoji, M., Hirai, S., Furiya, Y., Endoh, R., Mori, H., 1997. Amyloid beta protein 42 (43) in cerebrospinal fluid of patients with Alzheimer's disease. J. Neurol. Sci. 148, 41–45.

Tamaoka, A., Sawamura, N., Odaka, A., Suzuki, N., Mizusawa, H., Shoji, S., Mori, H., 1995. Amyloid beta protein 1–42/43 (Abeta 1–42/43) in cerebellar diffuse plaques: enzyme-linked immunosorbent assay and immunocytochemical study. Brain Res. 679, 151–156.

The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997. Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. Neurobiol. Aging 18, S1–S2.

Vassar, R., 2004. BACE1: the beta-secretase enzyme in Alzheimer's disease. J. Mol. Neurosci. 23, 105–114.

Vassar, R., Bennett, B.D., Babu-Khan, S., 1999. β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 735–741.

Yang, L.B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X.L., Beach, T., Sue, L., Wong, P., Price, D., Li, R., Shen, Y., 2003. Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat. Med. 9, 3–4.

Zhao, J., Paganini, L., Mucke, L., Gordon, M., Refolo, L., Carman, M., Sinha, S., Oltersdorf, T., Lieberburg, I., McConlogue, L., 1996. Beta-secretase processing of the beta-amyloid precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. J. Biol. Chem. 271, 31407–31411.

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Sustained progression and loss of the gender-related difference in atherosclerosis in the very old: A pathological study of 1074 consecutive autopsy cases

Motoji Sawabe[a,*], Tomio Arai[a], Ichiro Kasahara[a], Akihiko Hamamatsu[a], Yukiyoshi Esaki[a,1], Ken-ichi Nakahara[b,2], Kazumasa Harada[b], Kouji Chida[b], Hiroshi Yamanouchi[b], Toshio Ozawa[b], Kaiyo Takubo[c], Shigeo Murayama[d], Noriko Tanaka[e]

[a] *Department of Pathology, Tokyo Metropolitan Geriatric Hospital, 35-2 Sakae-cho, Itabashi, Tokyo 173-0015, Japan*
[b] *Department of Internal Medicine, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan*
[c] *Human Tissue Research Group, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*
[d] *Geriatric Neuroscience Research Group, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*
[e] *Department of Clinical Bioinformatics, Graduate School of Medicine, Tokyo University, Tokyo, Japan*

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Abstract

Introduction: Epidemiological surveys show decrease or reversal of male predominance in cardiovascular mortality in the very old, but the actual condition of atherosclerosis in the very old is largely unknown. The objective of this paper is to reveal whether the atherosclerosis continues to progress, or the gender-related difference exists in the very old.
Methods: The subjects were 1074 consecutive autopsy cases of in-hospital death. The male:female ratio was 1.1:1 and the average age was 80 years. Macroscopic evaluation was performed on the degree of atherosclerosis in 10 arteries including the intracranial arteries, carotid artery, aorta, coronary artery, and femoral artery.
Results: The severity of atherosclerosis differed greatly among arteries. The age-related increase of the atherosclerotic degree was evident, even after 80 years of age. The atherosclerosis was more severe in males than in females in their 60s, but this male predominance decreased with ageing and finally disappeared in their 90s.
Conclusion: The sustained progression of atherosclerosis and loss of the gender-related difference probably account for the increase of cardiovascular mortality in very old females. They also suggest that the prevention of the atherosclerotic progression is still important in the seventh and eighth decade of life.

Keywords: Aging; Atherosclerosis; Pathology; Autopsy; Gender-difference

1. Introduction

Atherosclerosis causes a large number of complications, such as cerebrovascular disease, coronary heart disease, ischemic bowel disease, renovascular hypertension, Leriche syndrome, peripheral arterial disease, and aneurysms. Chronic disseminated intravascular coagulation and elevated inflammatory markers, such as highly sensitive C-reactive protein, are reported to be other manifestations of atherosclerosis. Atherosclerosis, however, is also a sub-

Abbreviations: CHD, coronary heart disease; CSI, coronary stenotic index; CT, computer tomography; ICAI, intracranial atherosclerotic index; JG-SNP, the Japanese SNP database for geriatric research; MRI, magnetic resonance imaging; PAI, pathological atherosclerotic index

* Corresponding author. Tel.: +81 3 3964 1141; fax: +81 3 3964 1982.
E-mail address: sawabe@tmig.or.jp (M. Sawabe).
[1] Present address: Department of Pathology, Sekishinkai Sayama Hospital, Saitama, Japan.
[2] Present address: Medical Division, Nagasaki Medical Center, Omura, Japan.

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clinical entity because it does not always result in these complications even in cases with severe atherosclerosis. It is, therefore, difficult to speculate on the severity of atherosclerosis only from the presence of these complications. The clinical assessment of atherosclerosis is a challenging subject and the pathological study by autopsy is still the most reliable assessment method of systemic atherosclerosis.

Although several pathological studies have been reported regarding the severity of atherosclerosis [1–8], very little information was available about atherosclerosis in the elderly, especially in people of more than 80 years of age. It is still unknown whether atherosclerosis continues to progress in the very old, or whether the gender-related difference exists in the elderly because decades have passed since the menopause in female subjects. Recent epidemiological surveys showed decrease or reversal of the male predominance in cardiovascular mortality in the very old both in Japan and the U.S. [9,10], but the exact cause was unspecified. To address these issues, we performed a comprehensive pathological study in more than 1000 consecutive autopsy cases. This is the first pathological report analyzing the gender-specific, age-related changes of atherosclerosis in the very elderly.

2. Subjects and methods

2.1. Subjects

The subjects were 1074 consecutive elderly autopsy cases performed at Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan from 1995 to 2000. The details of the subjects including major clinical diagnosis and direct causes of death examined by autopsy are summarized in Table 1. We have been presenting our autopsy cases on an Internet-based database on the web since April 2003, which was named "The Japanese SNP database for geriatric research (JG-SNP)" located at http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html, as was previously reported [11]. The JG-SNP included all the subjects used in this study. The subjects did not include any medicolegal cases.

Tokyo Metropolitan Geriatric Hospital is a community-based general hospital for the aged and has all medical departments except Obstetrics and Pediatrics. Over 90% of the outpatients come from the neighboring wards or cities of Tokyo. The average day of hospitalization had decreased during the observation period from 45.6 days in 1995 to 24.3 days in 2000. The average autopsy rate in this period was 40% in whom the brain was available in 85%.

2.2. Pathological assessment of atherosclerosis

The method of the pathological assessment of atherosclerosis was recently reported [12]. Briefly, the varying degree of atherosclerosis in eight large arteries was evaluated by macroscopic examination of the luminal surface in the formalin-fixed arteries. The eight large arteries included the common carotid artery, subclavian artery, aorta, splenic artery, superior mesenteric artery, common iliac artery, external iliac artery, and left femoral artery. The atherosclerotic degree was scored according to the ratio of the occupying atheroma to the entire intimal area: from 0 (absent, less than 1/20 of the intimal areas occupied by the atheroma), 2 (minimal, 1/20–1/6), 4 (mild, 1/6–1/3), 6 (moderate, 1/3–2/3) to 8 (severe, 2/3–1). A comparison was made to the standard grading method proposed by the American Heart Association in 1968 [13]. The grading panel of AHA and corresponding scores in our scale are shown in Fig. 1.

The pathological atherosclerotic index (PAI) was defined as the average atherosclerotic degree of these eight arteries. The coronary stenotic index (CSI) was studied according to the previous report [14]. Examination of the coronary scle-

Table 1
Clinical summary of the patients

Gender	Males	Females
Number of cases	565	509
Age at death (year)		
45–59	5	7
60–69	63	41
70–79	220	144
80–89	217	203
90–104	60	114
Mean age[a]	79.2 ± 8.1 (52–102)	81.8 ± 9.5 (47–104)
Body mass index (kg/m^2)[a]	16.9 ± 3.4 (10.7–28.4)	17.2 ± 4.0 (8.4–37.9)
History of smoking	382/519 (73.6%)	98/445 (22.0%)
Clinical diagnoses (%)		
Cerebrovascular disease[b]	187 (33.1%)	157 (30.8%)
Coronary heart disease[c]	96 (17.0%)	77 (15.1%)
Aneurysm	27 (4.8%)	17 (3.3%)
Peripheral arterial disease	24 (4.2%)	17 (3.3%)
Hypertension	131 (23.2%)	134 (26.3%)
Diabetes mellitus	78 (13.8%)	63 (12.4%)
Hyperlipidemia	11 (1.9%)	6 (1.2%)
Direct causes of death (%)[d]		
Cardiovascular events	70 (15.3%)	76 (18.3%)
Cerebral infarctions or hemorrhages	14 (3.1%)	24 (5.8%)
Myocardial infarction	43 (9.4%)	37 (8.9%)
Other events[e]	13 (2.8%)	15 (3.6%)
Pneumonia	141 (30.8%)	71 (17.1%)
Malignancy	160 (34.9%)	138 (33.3%)

[a] The figures are the averages ± S.D. and ranges in parentheses.
[b] The clinical diagnosis of cerebrovascular disease was based on neurological signs and symptoms usually with radiological evidences, which includes transient ischemic attack, reversible ischemic neurological deficit, subarachnoid hemorrhage, cerebral hemorrhage, and cerebral infarction.
[c] The coronary heart disease includes angina pectoris and myocardial infarction.
[d] The direct causes of death are determined by autopsy findings of 872 cases (458 males and 414 females) among the subjects.
[e] The other cardiovascular events include aneurysmal rupture, intestinal infarction, and severe peripheral arterial disease.

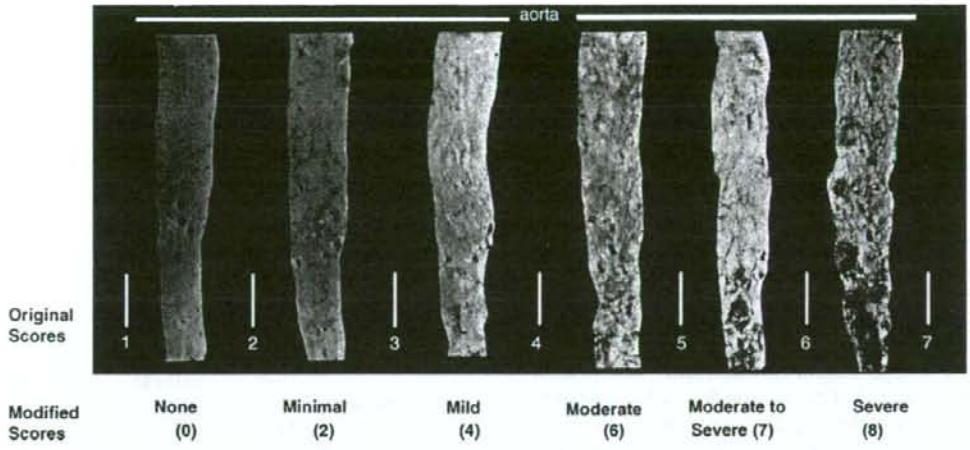

Fig. 1. Grading panel for atherosclerotic lesions, issued by the Committee on grading lesion, Council of Atherosclerosis, American Heart Association. The photographs show the aorta with different grades of atherosclerosis. Corresponding modified scores of our scale are shown beneath the original scores from 0 to 8. Reprint from the article by McGill et al. [13] with permission.

rosis was made by transverse section at 5 mm intervals. The degree of coronary stenosis was scored from 0 to 5: 0 in no sclerosis, 1 in slight stenosis, 2 in 25% stenosis, 3 in 50%, 4 in 75%, and 5 in 100% obstruction. The CSI was the sum of the stenotic scores of the three branches; left anterior descending branch, left circumflex branch, and right coronary artery. The intracranial atherosclerotic index (ICAI) was examined as previously reported [15]. The cut sections of the intracranial arteries were observed and the degree of stenosis was scored from 0 to 3: 0 in no stenosis, 0.5 in the cases only with fatty streaks, 1 in less than 50% stenosis, 2 in 50% to 90% stenosis, and 3 in 90% stenosis to occlusion. The ICAI was the sum of the stenotic scores of the left and right middle cerebral arteries and basilar artery.

2.3. Interobserver and intraobserver variations

The Bland–Altman analysis was performed for statistical analysis of interobserver and intraobserver variations, as shown in Fig. 2 [16]. The assessment was performed by two of the authors on 180 arteries derived from 15 cases. The mean difference was 0.5 (95% confidence interval; 0.3–0.7), while the upper and lower 95% limits of agreement were 3.3 (3.3–3.7) and −2.3 (−2.7 to −2.0), respectively. The standard deviation of intraobserver differences was 1.3. Considering the range from zero to eight of the atherosclerotic degree, the interobserver and intraobserver variations seemed acceptable.

2.4. Statistical analysis

A Mann–Whitney test was performed to compare the atherosclerotic degrees in the individual arteries between genders. The interobserver and intraobserver variations were assessed by the Bland and Altman plot analysis [16]. The difference between the atherosclerotic degrees assessed by the different observer against their mean was plotted to examine the interobserver variation. Repeatability was similarly

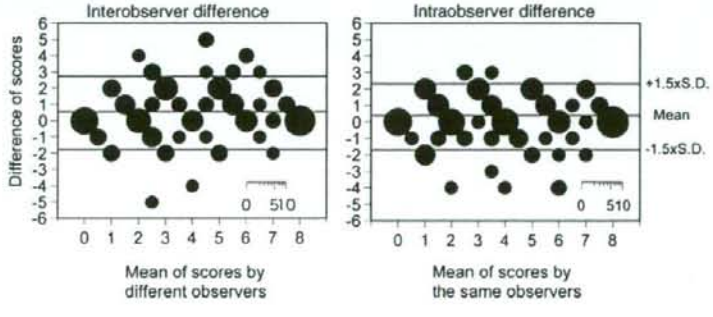

Fig. 2. Bland–Altman plot for statistical analysis of interobserver and intraobserver differences. The assessment was performed on 180 arteries derived from 15 cases. The areas of the circles represent the number of the cases.

assessed by plotting the difference between the atherosclerotic degrees assessed by the same observer against their mean. The statistical significance level was set at 0.05. The SAS system for Windows (Version 8.1) and JMP (Version 5.1) (SAS Institute Inc., NC) were used for the statistical analyses.

2.5. Ethical considerations

Written informed consent was obtained from the bereaved family of each of the patients prior to the autopsy examination. The use of autopsy materials for medical education and research is generally permitted by the Act of Postmortem Examinations of Japan.

3. Results

3.1. Distribution of the atherosclerotic degrees

Fig. 3 shows the distribution of the atherosclerotic degrees of the eight large arteries, PAI, CSI, and ICAI. The medians were high in the common iliac artery and aorta, while they were low in the splenic and superior mesenteric arteries. Variations in the degree of atherosclerosis were large in the external iliac and femoral arteries. The average (±S.D.) of the PAI, CSI, and ICAI were 3.9 (±1.5), 8.2 (±3.5), and 2.8 (±2.1), respectively. The atherosclerotic degree of the subclavian artery, PAI, and CSI followed a Normal distribution (Gaussian).

3.2. Gender-specific, age-related changes of the atherosclerotic degrees

The age-related increase in the degree of atherosclerosis was evident in both genders, as shown in Fig. 4. The atherosclerotic degree is the highest in the aorta, followed by the femoral artery, common carotid artery, and superior mesenteric artery. For subjects in their 60s and 70s, the degree of atherosclerosis is statistically higher in males than in females in most arteries, but no statistical differences are observed when subjects reach their 90s. Namely, the gender-related differences subsequently reduced with ageing and finally disappeared in their 90s. The changing rate of the age-related increase in the degree of atherosclerosis, namely the slope of the line graphs, was especially high in the femoral artery.

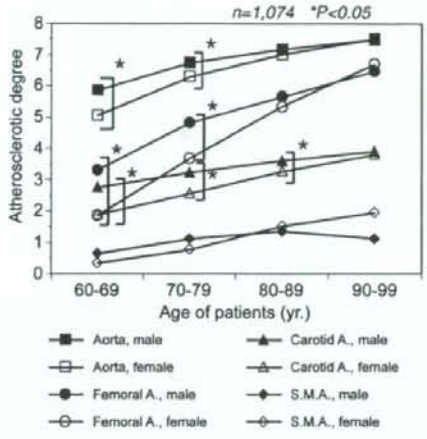

Fig. 4. Gender-specific, age-related changes of the degree of atherosclerosis in the individual arteries. S.M.A.; superior mesenteric artery. (■) Aorta, male; (□) aorta, female; (●) femoral A., male; (○) femoral A., female; (▲) carotid A., male; (△) carotid A., female; (♦) S.M.A., male; (◊) S.M.A., female.

The average CSI was statistically higher in males than in females in their 60s and 70s, as shown in Fig. 5. The age-

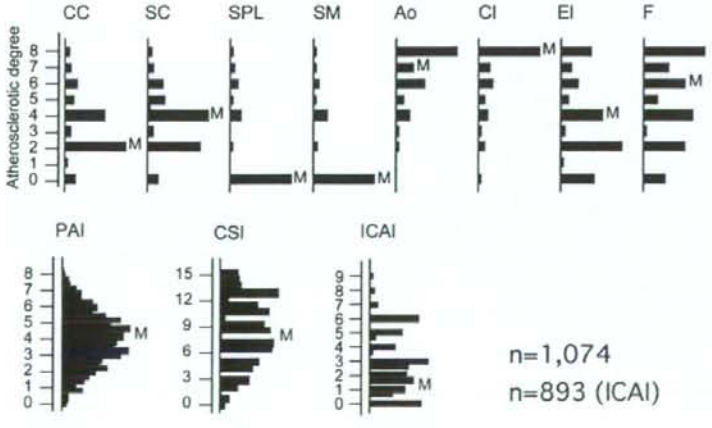

Fig. 3. Severity of atherosclerosis in the individual arteries. Ao, aorta; CC, common carotid artery; CI, common iliac artery; CSI, coronary stenotic index; EI, external iliac artery; F, femoral artery; ICAI, intracranial atherosclerotic index; M, median of the atherosclerotic degrees or indices; PAI, pathological atherosclerotic index; SC, subclavian artery; SM, superior mesenteric artery; SPL, splenic artery.

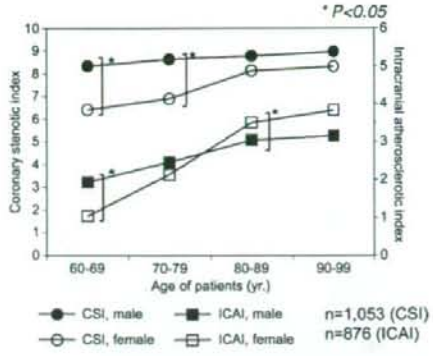

Fig. 5. Gender-specific, age-related changes of the coronary stenotic index (CSI) and intracranial atherosclerotic index (ICAI). (●) CSI, male; (○) CSI, female; (■) ICAI, male; (□) ICAI, female.

related increase of ICAI was larger in females than in males. In their 80s, the ICAI was statistically even higher in females than in males.

4. Discussion

4.1. Distribution of the atherosclerotic degrees

The present study revealed the severity of atherosclerosis differed by the arterial segments. The aorta and arteries of the lower extremities were severely affected, while the abdominal arteries, such as the splenic and superior mesenteric arteries, were mildly affected. These results were consistent with previous reports stating the abdominal aorta and common iliac arteries were the most severe sites of atherosclerotic involvement [1,2,4].

4.2. Gender-specific, age-related changes of the atherosclerotic degrees

Atherosclerosis continued to progress in the elderly in most arteries except for the coronary artery in males. Males in their 60s were more severely affected by atherosclerosis than age-matched females, but this gender-related difference reduced with ageing and finally disappeared in subjects in their 90s. In the case of the intracranial arteries and superior mesenteric artery, atherosclerosis was even more severe in females than in males in their 80s or 90s. The loss of the gender difference of atherosclerosis in the very old seems to influence the mortality. In Japan, the mortalities from heart diseases and cerebrovascular diseases are higher in males than in females in all age groups [9] and the gender differences in mortality increase with ageing, peak at 85–89 years and decrease thereafter in both diseases. A similar trend is present in the death rates from heart diseases in the U.S. [10]. The death rates from cerebrovascular diseases in the U.S. are almost even in each age group except for that of 85 years and over, in whom female predominance is evident. The loss of the gender-related difference in atherosclerosis in the very old seems responsible for the decrease or reversal of male predominance in cardiovascular mortality in the very old. Our results also suggest that the prevention of the atherosclerotic progression is still necessary in the very old, especially in females.

4.3. Limitations of the study

The subjects of this study were autopsy cases of the patients in a geriatric hospital. Therefore, we need to evaluate whether the subjects represent a hospitalized population or the demographics in Japan. The general prevalence of the major underlying diseases over 70 years of age was as follows: 29.5% in males versus 28.5% in females in hypertension (systolic blood pressure $\geq$160 Torr or diastolic blood pressure $\geq$95 Torr), 11.6% versus 21.3% in diabetes mellitus (under diabetic control or HbA1c $\geq$ 6.1%), and 15.8% versus 38.5% in hyperlipidemia (total cholesterol $\geq$ 220 mg/dl) [17]. The figures were similar to those of our results except for hyperlipidemia. Considering the low average BMI of the subjects (17 kg/m^2), the undernourished conditions seemed to contribute to the low incidence of hyperlipidemia. The death rates per 100,000 over 75 years of age in Japan were as follows: 1027.6 in males versus 860.1 in females in the cerebrovascular diseases, 537.9 versus 398.5 in ischemic heart disease, 1017.6 versus 571.4 in pneumonia, and 2085.7 versus 980.0 in malignancy [9]. The frequency of cerebrovascular diseases seems higher than our autopsy data. Since the subjects comprised entirely of the Japanese race except for one Korean and the national health insurance covers the whole population in Japan, the selection bias from the racial and socioeconomic differences of the subjects seems minimal. Altogether our subjects may represent the general population in Japan.

4.4. Perspectives

Atherosclerosis is a multifactorial disease and 30–66% of the variation of the atherosclerosis could be explained by the genetic factors [18–20]. In this context, the roles of the genetic polymorphism have been extensively studied to identify the most responsible genes for atherosclerosis. Since the postmortem pathological evaluation of the atherosclerosis is more accurate than other clinical methods, a few autopsy studies have been conducted for the genetic studies, including Helsinki Sudden Death Study [21,22] and Pathological Determinants of Atherosclerosis in Youth Study [23,24]. Both studies examined the aortic and coronary atherosclerosis in large numbers of forensic autopsy cases of sudden death and analyzed the correlations between the genetic polymorphism and the pathologically verified atherosclerosis. We also have been engaged in the researches of the genetic polymorphism of atherosclerosis, based on the autopsy cases used in this study. In the course of our study, we have obtained several

interesting results, including those appeared in this paper. We hope to identify relevant genetic polymorphic sites of the candidate genes for atherosclerosis.

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References

[1] Roberts JC, Moses C, Wilkins RH. Autopsy studies in atherosclerosis. I. Distribution and severity of atherosclerosis in patients dying without morphologic evidence of atherosclerotic catastrophe. Circulation 1959;20:511–9.

[2] Roberts JC, Wilkins RH, Moses C. Autopsy studies in atherosclerosis. II. Distribution and severity of atherosclerosis in patients dying with morphologic evidence of atherosclerotic catastrophe. Circulation 1959;20:520–6.

[3] Strong JP, McGill Jr HC. The natural history of aortic atherosclerosis: relationship to race, sex, and coronary lesions in New Orleans. Exp Mol Pathol 1963;52(1):15–27. Suppl.

[4] Solberg LA, McGarry PA, Moossy J, Strong JP, Tejada C, Löken AC. Severity of atherosclerosis in cerebral arteries, coronary arteries, and aortas. Ann NY Acad Sci 1968;149:956–73.

[5] Bjurulf P. Atherosclerosis in different parts of the arterial system. Am Heart J 1964;68:41–50.

[6] Reiner L, Jimenez FA, Rodriguez FL. Atherosclerosis in the mesenteric circulation. Observations and correlations with aortic and coronary atherosclerosis. Am Heart J 1963;66:200–9.

[7] Larsen E, Johansen AA, Andersen D. Gastric arteriosclerosis in elderly people. Scand J Gastroenterol 1969;4(4):387–9.

[8] Järvinen O, Laurikka J, Sisto T, Salenius J-P, Tarkka MR. Atherosclerosis of the visceral arteries. Vasa 1995;24:9–14.

[9] Statistics and Information Department, Ministry of Health, Labour and Welfare, Japan. Vital Statistics, Abridged Life Tables for Japan; 2003. Available at http://www.mhlw.go.jp/english/database/db-hw/index.html (in English) or http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai03/index.html (in Japanese). Accessed on July 10, 2005.

[10] U.S. Census Bureau. Statistical Abstract of the United States 2004–2005, Section 2. Vital Statistics, Table No. 107 and 108; 2005. Available at: http://www.census.gov/prod/www/statistical-abstract-03.html. Accessed on July 10, 2005.

[11] Sawabe M, Arai T, Kasahara I, et al. Development of a geriatric autopsy database and internet-based database of Japanese single nucleotide polymorphisms for geriatric research (JG-SNP). Mech Ageing Dev 2004;125:547–52.

[12] Sawabe M, Takahashi R, Matsushita S, et al. Aortic pulse wave velocity and the degree of atherosclerosis in the elderly: a pathological study based on 304 autopsy cases. Atherosclerosis 2005;179:345–51.

[13] McGill Jr HC, Brown BW, Gore I, et al. Report of Committee on grading lesions, council on arteriosclerosis, American Heart Association. Grading human atherosclerotic lesions using a panel of photographs. Circulation 1968;37:455–9.

[14] Chida K, Ohkawa S, Watanabe C, Shimada H, Ohtsubo K, Sugiura M. A morphological study of the normally aging heart. Cardiovasc Pathol 1994;3:1–7.

[15] Koyama S, Saito Y, Yamanouchi H, et al. Marked decrease of intracranial atherosclerosis in contrast with unchanged coronary artery stenosis in Japan. Nippon Ronen Igakkai Zasshi (Jpn J Geriat) 2003;40:267–73 (in Japanese with English abstract).

[16] Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1:307–10.

[17] Ministry of Health, Labour and Welfare, Japan. The National Nutrition Survey in Japan. Tokyo: Dai-ichi Shuppan Publishing Co. Ltd.; 2004 (in Japanese).

[18] Duggirala R, Villalpando CG, O'Leary DH, Stern MP, Blangero J. Genetic basis of variation in carotid artery wall thickness. Stroke 1996;27:833–7.

[19] Zannad F, Visvikis S, Gueguen R, et al. Genetics strongly determines the wall thickness of the left and right carotid arteries. Hum Genet 1998;103(2):183–8.

[20] O'Donnell CJ, Chazaro I, Wilson PWF, et al. Evidence for heritability of abdominal aortic calcific deposits in the Framingham Heart Study. Circulation 2002;106(3):337–41.

[21] Ilveskoski E, Perola M, Lehtimäki T, et al. Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. Circulation 1999;100(6):608–13.

[22] Pöllänen PJ, Lehtimäki T, Mikkelsson J, et al. Matrix metalloproteinase 3 and 9 gene promoter polymorphisms: joint action of two loci as a risk factor for coronary artery complicated plaques. Atherosclerosis 2005;180(1):73–8.

[23] Strong JP, Malcom GT, McMahan CA, et al. Prevalence and extent of atherosclerosis in adolescents and young adults: implications for prevention from the pathobiological determinants of atherosclerosis in youth study. JAMA 1999;281(8):727–35.

[24] Scheer WD, Boudreau DA, Hixson JE, et al. ACE insert/delete polymorphism and atherosclerosis. Atherosclerosis 2005;178(2):241–7.

SHORT REPORT

Selective loss of Purkinje cells in a patient with anti-glutamic acid decarboxylase antibody-associated cerebellar ataxia

Kazuyuki Ishida, Hiroshi Mitoma, Yoshiaki Wada, Teruaki Oka, Junji Shibahara, Yuko Saito, Shigeo Murayama, Hidehiro Mizusawa

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Anti-glutamic acid decarboxylase antibody is associated with the development of progressive cerebellar ataxia and slowly progressive insulin-dependent diabetes mellitus. Previously, the neurophysiological characteristics of IgG in the cerebrospinal fluid of a patient with anti-glutamic acid decarboxylase antibody-associated progressive cerebellar ataxia and slowly progressive insulin-dependent diabetes mellitus were reported. Using a voltage-gated whole-cell recording technique, it was observed that the IgG in the cerebrospinal fluid of the patient selectively suppressed the inhibitory postsynaptic currents in the Purkinje cells. The patient died from aspiration pneumonia. Postmortem examination showed almost complete depletion of the Purkinje cells with Bergmann gliosis. Therefore, the main cause of cerebellar ataxia observed in this case may be attributed to the near-complete depletion of the Purkinje cells. In this paper, the pathomechanisms underlying Purkinje cell damage are discussed.

Glutamic acid decarboxylase (GAD) is a catalytic enzyme that converts glutamic acid to γ-aminobutyric acid, a major inhibitory neurotransmitter. A disease group that is characterised by the presence of a circulating autoantibody against GAD (anti-GAD antibody) includes the following: slowly progressive insulin-dependent diabetes mellitus (SPIDDM), stiff-person syndrome (SPS) and progressive cerebellar ataxia (PCA).[1–3] Anti-GAD antibody is one of the serological diagnostic markers of these diseases. Honnorat *et al*[4] reported a significant link between the anti-GAD antibody and cerebellar ataxia after screening 9000 serum samples. In addition, autoimmune mechanisms against GAD are presumed to be the causative agents of these diseases.[5] Here, we report the autopsy findings of PCA with anti-GAD antibody and discuss the pathomechanism of this rare disease.

CASE REPORT

We previously reported part of the clinical course of a patient with PCA and SPIDDM, and showed the neurophysiological characteristics of IgG in the cerebrospinal fluid.[6] In September 1996, a 66-year-old woman developed cerebellar ataxia of the limbs and trunk. In April 1997, she had sudden onset of hyperglycaemia, and was subsequently diagnosed with anti-GAD-associated SPIDDM. In May 1997, she was bedridden due to severe cerebellar ataxia; other symptoms such as extrapyramidal or pyramidal tracts were not observed. The patient was diagnosed with anti-GAD antibody-associated PCA, and received four rounds of plasma exchange and immunosuppressive treatment. After treatment, the patient showed slight improvement in cerebellar ataxia.

In December 2000, the patient experienced painful spasms and rigidity in the trunk that mimicked symptoms of SPS. Diazepam and baclofen were effective in ameliorating the severe pain associated with the spasms and rigidity. The painful spasms subsided spontaneously within 2 months. The patient died of aspiration pneumonia in October 2001.

During the 5-year clinical course, repeated neuroradiological examinations showed no significant cerebellar atrophy. Using a voltage-gated whole-cell recording technique, we observed that the IgG in the cerebrospinal fluid of the patient, selectively suppressed the inhibitory postsynaptic currents in the Purkinje cells.[6 7]

Postmortem examination

Postmortem examination was performed 22 h after death. The brain weighed 1150 g. The brain and the entire spinal cord were fixed in formalin and prepared for a morphological examination. Macroscopically, there was no atrophy of the cerebrum, brain stem, cerebellum (fig 1A) and spinal cord. The representative areas were examined by routine and immunohistochemical staining, as reported previously.[8] In short, 6-μm thick serial sections were stained with haematoxylin and eosin, Klüver–Barrera and Bodian silver staining. For the immunohistochemical study, 6-μm dewaxed and microwave-irradiated sections were stained using a Ventana 20NX automatic stainer (Ventana, Tucson, Arizona, USA). Microscopical examination showed almost complete depletion of the Purkinje cells and diffuse proliferation of the Bergmann glia (fig 1B). The number of remaining Purkinje cells was no more than one per cerebellar folium. Bodian staining showed multiple empty baskets (fig 1C). There was no specific inflammatory response, and the other structures of the central nervous system, including the cerebral cortex, white matter, basal ganglia, brain stem and spinal cord, did not show marked pathological changes. The pancreas showed a definite and marked decrease in the islets in the tail (fig 1D), and lymphocytic infiltration in the islets situated in the pancreatic body.

DISCUSSION

The selective loss of both Purkinje cells and pancreatic islets was a characteristic finding in this case. The selective degeneration of the Purkinje cells partially mimics the pathological changes observed in paraneoplastic cerebellar ataxia associated with anti-mGluR1 or anti-Yo antibody; however, the exclusive pathological changes related to the Purkinje cells constitute a unique feature of this case.[9 10] On the other hand, the lymphocytic infiltration in the pancreas and the selective decrease in the pancreatic islets corresponded with the pathological findings of autoimmune insulin-dependent diabetes mellitus.[11] Therefore, the main causes of cerebellar ataxia and diabetes mellitus seem to be related to the depletion of the Purkinje cells and the decrease in the pancreatic islets.

Abbreviations: GAD, glutamic acid decarboxylase; PCA, progressive cerebellar ataxia; SPIDDM, slowly progressive insulin-dependent diabetes mellitus; SPS, stiff-person syndrome

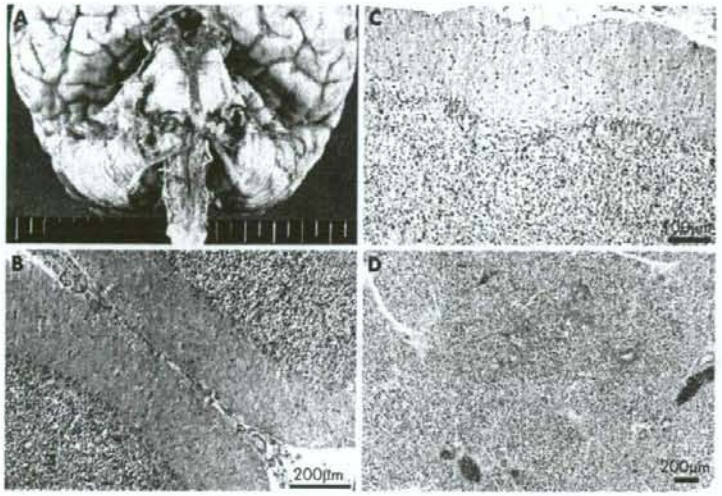

Figure 1 (A) Macroscopic appearance of the brain stem and cerebellum. There are no atrophic changes in the cerebellum and brain stem. (B) Haematoxylin and eosin staining of the cerebellar cortex. There is severe depletion of Purkinje cells and proliferation of Bergmann glia. (C) Bodian staining of the cerebellar cortex. Multiple empty baskets can be observed. (D) Pancreatic tail (haematoxylin and eosin staining). There is a selective decrease in the pancreatic islets.

respectively. To our knowledge, this is the first autopsy report of PCA associated with anti-GAD antibody.

Immunohistochemical staining using anti-GAD and anti-calbindin antibodies failed to react with the patient's specimen; this indicated a complete loss of antigenicity in the patient's specimen, due to postmortem delay and excessive fixation. Therefore, it became difficult to analyse the morphological changes in the other GAD-containing neurones, such as the cerebellar basket cells and the spinal Renshaw cells. However, the existence of multiple empty baskets suggested that, in contrast to the Purkinje cells that were lost, the basket cells were relatively preserved.[12]

We inferred two possible pathomechanisms to explain the Purkinje cell damage: indirect and direct immune-mediated mechanisms. The indirect mechanism might be associated with excitotoxicity of the Purkinje cells by the selective suppression of inhibitory postsynaptic currents and the attenuation of inhibition of excitatory postsynaptic currents by the anti-GAD antibody.[6 7 11] The direct mechanism might be mediated by cytotoxic reactions against the Purkinje cells caused by the invading leucocytes, as observed in the pancreatic islets. However, it is presently unclear whether the mechanisms that are more likely to have caused the Purkinje cell damage are indirectly or directly immune-mediated.

The patient experienced painful muscle spasms that mimic symptoms of SPS. The muscle spasms observed in SPS are considered to occur as a result of the dysfunction of the Renshaw cells that are γ-aminobutyric acid inhibitory interneurones in the spinal cord.[14] Various pathological changes are observed in the spinal cord of patients with SPS; however, lymphocytic cuffing and a decrease in the number of anterior horn neurones are considered to be representative of SPS.[15] In contrast, the pathological changes observed in our patient were unremarkable; this suggests that the Renshaw cells were not severely damaged. This may explain the transient nature of the muscular spasms in this case.

Based on the quantitative analysis of the brain autopsy of a patient with SPS and without cerebellar ataxia, Warich-Kirches *et al*[16] reported diminished cell density of the inhibitory neurones in the cerebellar cortex. Combining their case results with ours might show the phenotypic overlap of the anti-GAD autoimmunity-associated neurological diseases.

Authors' affiliations
Kazuyuki Ishida*, Department of Neurology, Tamagawa Hospital, Setagaya-ku, Tokyo, Japan
Hiroshi Mitoma, Mitoma Neurological Clinic, Shinjuku-ku, Tokyo, Japan
Yoshiaki Wada, Department of Rehabilitation, Tamagawa Hospital, Setagaya-ku, Tokyo, Japan
Teruaki Oka, Department of Pathology, Kanto Central Hospital, Setagaya-ku, Tokyo, Japan
Junji Shibahara, Department of Pathology, University of Tokyo, Bunkyo-ku, Tokyo, Japan
Yuko Saito, Shigeo Murayama, Department of Neuropathology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan
Hidehiro Mizusawa, Department of Neurology and Neurological Science, Tokyo Medical and Dental University Graduate School, Bunkyo-ku, Tokyo, Japan

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Informed consent was obtained from the family of the patient for the publication of her details in this paper.

Correspondence to: Dr Kazuyuki Ishida, Institute of Oriental Medicine, Tokyo Women's Medical University, School of Medicine, 4th floor, Shinjuku NS Building, 2-4-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 163-0804, Japan; k-ishida@iom.twmu.ac.jp

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REFERENCES

1 **Seissler J**, Amann J, Mauch L, *et al*. Prevalence of autoantibodies to the 65- and 67-kD isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 1993;**92**:1394–9.
2 **Saiz A**, Arpa J, Sagasta A, *et al*. Autoantibodies to glutamic acid decarboxylase in three patients with cerebellar ataxia, late-onset insulin-dependent diabetes mellitus, and polyendocrine autoimmunity. *Neurology* 1997;**49**:1026–30.
3 **Vianello M**, Tavolato B, Giometto B. Glutamic acid decarboxylase autoantibodies and neurological disorders. *Neurol Sci* 2002;**23**:145–51.
4 **Honnorat J**, Saiz A, Giometto B, *et al*. Cerebellar ataxia with anti-glutamic acid decarboxylase antibodies: study of 14 patients. *Arch Neurol* 2001;**58**:225–30.
5 **Dalakas MC**, Li M, Fujii M, *et al*. Stiff person syndrome: quantification, specificity, and intrathecal synthesis of GAD65 antibodies. *Neurology* 2001;**57**:780–4.
6 **Ishida K**, Mitoma H, Song SY, *et al*. Selective suppression of cerebellar GABAergic transmission by an autoantibody to glutamic acid decarboxylase. *Ann Neurol* 1999;**46**:263–7.