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Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients

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Objectives – To investigate a possible implication of inflammatory processes in the development of dementia in cerebrovascular disease. *Patients and methods* – We examined the levels of interleukin-6 (IL-6) in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) ($n = 26$), ischemic cerebrovascular disease without dementia (CVD) ($n = 11$), vascular dementia (VD) ($n = 11$), and other neurological disorders ($n = 21$) using sensitive enzyme-linked immunosorbent assay. *Results* – The CSF concentrations of IL-6 were significantly elevated in patients with VD compared with those of patients with AD or CVD. *Conclusion* – The CSF IL-6 levels are increased in patients with VD, suggesting that inflammatory mechanisms may be involved in the development of cognitive decline in some patients with cerebrovascular disease. CSF IL-6 may be a biological marker for dementia in cerebrovascular disease.

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Key words: vascular dementia; Alzheimer's disease; cerebrovascular disease; interleukin-6; cytokines; tau protein; cerebrospinal fluid; biological marker

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Vascular dementia (VD) is a common cause of dementia in Japan (1). However, the mechanisms of clinical cognitive deterioration in patients with cerebral ischemia are not completely understood. Several recent studies have provided insight into the possible role of inflammatory processes in the development of brain ischemia and multi-infarct cognitive impairment, as demonstrated by the accumulation of inflammatory cells and mediators in the ischemic brain (2–5). Vila et al. (6) reported that interleukin-6 (IL-6) participates in the acute-phase response that follows cerebral ischemia and that an association exists between high levels of IL-6 and early neurological worsening. A case-control genetic study reported a positive association of the -174 G/C IL-6 gene polymorphism and the risk of multi-infarct dementia (7). These data led the hypothesis that inflammatory mechanisms play a crucial role in the pathogenesis of the development of dementia in cerebrovascular disease. Cerebrospinal fluid (CSF) levels of IL-6 are elevated in central nervous system (CNS) infections and non-infectious CNS inflammatory diseases, indicating that levels of IL-6 in the CSF reflect the inflammatory processes (8–10). Little has been reported about the IL-6 levels in the CSF of patients with VD. Previous studies reported that these levels did not differ from those of controls (11–13). However, some patients with VD in the previous study investigated by Yamada et al. (13) showed high CSF concentrations of IL-6. In order to clarify the association of inflammatory mechanisms with VD, we examined the CSF levels of IL-6 in patients with VD as well as in patients with Alzheimer's disease (AD) and ischemic cerebrovascular disease without dementia (CVD).

Subjects and methods

The subjects were 26 patients with AD (mean age ± SD 66.8 ± 8.2 years), 11 patients with CVD (70.0 ± 6.2 years), 11 patients with VD (74.5 ± 4.5 years), and 21 patients with other neurological disease (OND) (68.4 ± 5.8 years). Assessments of these patients included a carefully examined medical history, physiological examination, drug inventory, neurological examination, comprehensive cognitive evaluation with the use of the Functional Assessment Staging of Alzheimer's disease (FAST staging), the Mini-Mental State Examination (MMSE), neuroimaging assessments of CT scan or MRI and single photon emission computed tomography of the head, and routine laboratory tests, such as blood analysis and biological

examination. Patients who satisfied the Diagnostic and Statistical Manual of Mental Disorders, third edition-revised (DSM-III-R) (14) and the diagnostic criteria of the National Institute of Neurological and Communicative Disorders Association (NINCDS-ADRDA) (15) and those scoring 4 points or less on Hachinski's ischemic score (16) were diagnosed as having AD. Patients who satisfied the DSM-III-R and the ADDTC criteria for ischemic VD (17) and those scoring 7 points or more on Hachinski's ischemic score were diagnosed as having VD. All the patients with VD showed stepwise deterioration of cognitive function and one or more infarcts outside the cerebellum detected by neuroimaging. The CVD group was defined as patients who had a history of stroke episode and with CT scan or MRI findings of infarcts without dementia. OND patients consisted of seven patients with Parkinson's disease (PD), four patients with amyotrophic lateral sclerosis (ALS), four patients with spinocerebellar degeneration, two patients with peripheral neuropathy, two patients with tension-type headache, one patient with myopathy and one patient with essential tremor. OND patients did not show any cognitive impairment. After informed consent from patients or their families, CSF was collected by lumbar puncture. CSF samples were stored at −80°C until assay. Collections of CSF from the patients with CVD or VD were performed during the chronic phase of the diseases when the progression of neurological deterioration was no longer observed. CSF IL-6 levels were determined in duplicate, using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Inc, Minneapolis, MN, USA). CSF total tau protein levels were measured using ELISA kit (Innogenetics, Gent, Belgium). Statistical significance was analyzed by one-way ANOVA, followed by *post hoc* tests. Correlation was analyzed by Spearman rank correlation test.

Results

As shown in Fig. 1, the concentrations of IL-6 in the CSF of VD patients were 5.67 ± 1.7 pg/ml (mean ± SE); those of patients with AD were 2.53 ± 0.87 pg/ml; those of patients with CVD were 2.15 ± 0.38 pg/ml; and those of patients with OND were 3.15 ± 0.67 pg/ml. Significantly elevated levels of IL-6 were found in the CSF of patients with VD compared with those in the CSF of patients with AD, CVD, and OND. There was no significant difference in CSF IL-6 levels between AD patients and CVD and OND patients. There were not a correlation between CSF IL-6 levels and

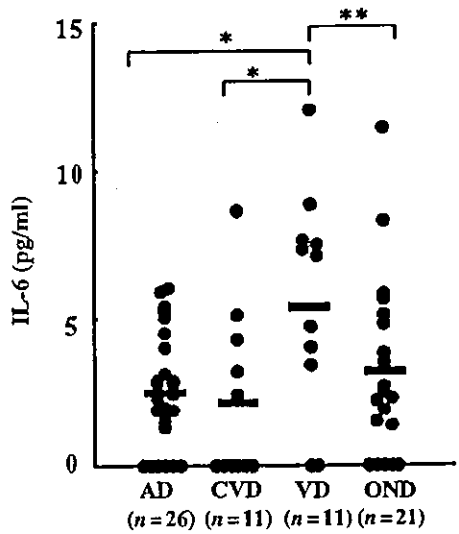

Figure 1. Interleukin-6 levels in the cerebrospinal fluid of the patients with Alzheimer's disease, ischemic cerebrovascular disease without dementia, vascular dementia, and other neurological disease. The horizontal bar indicates the mean level. Statistical differences were calculated using one-way ANOVA followed by a *post hoc* test; $*P < 0.01$, $**P < 0.05$.

Table 1 Total tau levels in cerebrospinal fluid

Disease	Mean ± SE (pg/ml)
AD	236.0 ± 17.2
CVD	115.0 ± 39.3
VD	116.8 ± 28.1
OND	126.4 ± 16.4

AD, Alzheimer's disease; CVD, cerebrovascular disease without dementia; VD, vascular dementia; OND, other neurological disorders.

MMSE scores in VD patients (data not shown). The levels of total tau protein in CSF were shown in Table 1. Significantly elevated levels of tau protein were found in the CSF of patients with AD compared with those in the CSF of patients with VD ($P < 0.01$). There were not significant differences between CSF tau levels in VD and those in CVD or OND.

Discussion

Interleukin-6 was described initially by Hirano (18) as a B cell differentiation factor usually derived from T cells and it can also be produced by astrocytes and microglia in the CNS (9, 19, 20). CSF levels of IL-6 were examined in patients with infectious or non-infectious inflammatory diseases of the CNS. CSF levels of IL-6 were also examined in patients with neurodegenerative disorders. In particular, controversial results ranging from no changed (21, 22), to increased (23) or decreased

(13) levels of IL-6 in the CSF have been reported in AD patients. Differences in sample size, selection of patients with AD and control subjects, or experimental procedures may account for these varying results. Our results show that there are no significant differences in the CSF levels of IL-6 between patients with AD and patients with CVD or OND. The OND group included patients with PD and ALS, in which higher levels of CSF IL-6 have been reported (23, 24). Indeed, the patients with the two highest levels in OND group in our study were a PD patient and an ALS patient, but CSF IL-6 levels were not altered in other patients with ALS or PD. We conclude that the levels of IL-6 are not altered in patients with AD, and that CSF IL-6 may not be a biological marker for the diagnosis of AD. On the contrary, we obtained significantly higher CSF levels of IL-6 in patients with VD, but not in patients with CVD who did not have dementia. However, previous reports indicated that CSF levels of IL-6 in VD patients were not significantly elevated (11, 12). It has been reported that patients with VD are heterogeneous and diagnosis criteria for VD are not interchangeable (25). Selection of patients may account for these differences. Not all the patients with VD showed higher levels of IL-6 in CSF in this study, but we used the DSM-III-R and ADTTC criteria for diagnosis of VD and employed probable cases in this study and these cases also showed significant lower levels of total tau protein in CSF compared with AD patients, suggesting that clinical diagnosis of VD patients was sufficient to segregate AD patients from VD cases. Recent reports indicated inflammatory process might be involved in cerebrovascular disease. Higher baseline levels of CSF IL-6 were shown to be related to early neurologic worsening in ischemic stroke, independent of the initial size topography or mechanism of ischemic infarction (6, 26). A genetic association of IL-6 polymorphism with multi-infarct dementia (7) and activation of the micoglia in Binswanger's disease, a form of VD has been shown (3). The increased intrathecal production of granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that stimulates microglial cell growth and has inflammatory properties, has been found in patients with VD (4). Taken together with our result, the inflammatory activations in the CNS might be associated with some part of VD patients and measurement of CSF IL-6 might provide a clue to differential diagnosis of dementia. Our study is a cross-sectional design, further studies using a longitudinal design with large samples are necessary to support these results.

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Soluble Aβ homeostasis in AD and DS: impairment of anti-amyloidogenic protection by lipoproteins

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Abstract

In order to assess whether lipoproteins are physiologically able to balance and modulate the sAβ homeostasis in vivo, soluble Aβ levels in lipoprotein-depleted plasma were measured as a function of age in normal controls, Alzheimer's disease (AD) patients, and Down's syndrome (DS) cases. The reshaping of sAβ homeostasis, in particular the sAβ42-lipoprotein interaction, takes place over normal-60's, whereas mild AD patients appear to have impaired this anti-amyloidogenic mechanism resulting in a significant increase of lipoprotein-free sAβ42. Similar loss of function takes place in Down's syndrome patients. Lipoprotein-free sAβ remains significantly elevated from the pre-symptomatic through the symptomatic stages of the disease, and declines with the progression of the AD-like pathology. The dissociation of sAβ from lipoprotein-particles also occurs in brain parenchyma and the presence of soluble dimeric lipoprotein-free Aβ prior to its parenchymal deposition in AD brains would support the hypothesis that functionally declined lipoproteins may be major determinants in the production of metabolic conditions leading to higher levels of sAβ species and cerebral amyloidosis.

Keywords: Alzheimer's disease; Down's syndrome; sAβ homeostasis; Lipoprotein-free Aβ; Aβ dimer

1. Introduction

Amyloid β (Aβ) is the major constituent of the fibrils deposited in senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) and Down's syndrome (DS). This peptide, originally thought not to be derived by normal processing of its precursor APP [30], is now known to be a normal soluble component (sAβ) of biological fluids [8,28,29,40] and brain parenchyma [24,33]. It has been reported that an increased amount of sAβ precedes the appearance of Aβ deposits in AD and DS brains [35] and accumulates with age [6], suggesting that sAβ species represent the direct precursors of the deposited fibrils. Ninety percent or more of the sAβ found in normal human plasma is associated with lipoprotein particles [20], which had been shown to maintain and stabilize the peptide solubility in vitro [19,21] and modulate its catabolism [8,20]. A recent study suggests that brain levels of total extractable Aβ becomes elevated very early in the disease process and correlates with measurable decline in cognitive functions [23], pointing to the in vivo sAβ homeostasis as a potential therapeutic target.

It is well known that aging is the most prevailing risk factor for sporadic AD patients. In vivo studies have shown that Aβ neurotoxicity is closely related to the aging brain via unknown age-related factors [7], perhaps reflecting metabolic alterations. Our previous experiments demonstrated that a functional decline for the physiologic

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protective role of apolipoproteins is detectable in patients with AD [20]. However, at which point in time normal metabolic conditions switch to those disease-related remains unknown, mainly due to the need of a large number of well-characterized cases in order to achieve statistical meaning. DS brains at different ages represent the various stages seen in AD pathology, [18,34], and plasma samples reconstructed from a large cohort of DS patients can mimic the pre-symptomatic and symptomatic AD-like metabolic conditions. We hypothesized that an alteration of the lipoprotein–sAβ interaction is able to initiate and/or maintain the cascade favoring Aβ oligomerization. To verify this hypothesis and extend previous observations [20,36], the effect of aging in the lipoprotein–sAβ interaction was examined in DS patients, age-matched normal controls (NCs) and AD cases. To further assess the in vivo relevance of lipoprotein-free brain sAβ in AD pathology, we have characterized the dissociation of sAβ from its lipidic environment in AD and control brain parenchyma.

2. Materials and methods

2.1. Patients

After informed consent was given, blood samples (7 ml) were collected in 0.1% EDTA from 178 Down's syndrome (DS; ages 1–64 years), 100 Alzheimer's disease (AD; ages 46–102 years), and 241 normal controls (NC; ages 6–105 years) after 12-h fasting. None of the individuals in either group had a history of liver disease or other condition that might have affected their lipoprotein profile and none were taking drugs known to affect lipid metabolism. DS patients were characterized through clinical evaluation; trisomy 21 was confirmed by chromosomal analysis. The degree of cognitive impairment was assessed with the Mini Mental State Examination (MMSE) [4]. Accordingly, AD patients were subgrouped into those with mild AD (MMSE, >20, n = 27), moderate AD (MMSE, 11–20, n = 29), or severe AD (MMSE, <11, n = 44).

2.2. Lipoprotein separation and depletion

After separation of plasma from blood cells, lipoprotein-depletion was carried out by preparative sequential density flotation ultracentrifugation using 600 μl of plasma and a protocol previously described [20] in 101 out of 178 patients with DS, 100 out of 100 patients with AD, and 103 out of 241 NCs. Briefly, the density of the collected plasma were adjusted to 1.25 g/ml using KBr and ultracentrifuged at 100,000 rpm for 8 h at 16 °C using a Hitachi RP100AT rotor. The infranatant at the density of 1.25 g/ml, named lipoprotein-depleted plasma (LPDP), as well as the floated lipoproteins were subjected to ultrafiltration using a 3 kDa cut-off membrane (Microcon 3; Amicon, Inc.) and stored until use either frozen or at 4 °C.

2.3. Tissue extraction

The AD brains used in this study were selected from patients that fulfilled the CERAD criteria [22] and classified on the basis of classic neuropathology (presence of senile plaques and neurofibrillary tangles). Gray matter was dissected free of vessels. Cerebral cortex (0.25 mg) from six AD and four control brains was homogenized with a motor-driven Teflon/glass homogenizer (20 strokes) in 1 ml of Tris-buffered saline (TBS, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl) and ultra-centrifuged using a Hitachi RP100AT rotor at 100,000 × g for 1 h. The resultant supernatant (named TBS soluble fraction) was subjected to size-exclusion chromatography and immunoblot, and sAβ species were quantitated via specific ELISAs. The pellet, after being washed once, was further extracted with 1 ml of 70% formic acid (FA) and the homogenate ultra-centrifuged as described above. The resultant supernatant (named FA soluble fraction) was also subjected to size-exclusion chromatography, immunoblot analysis, and ELISAs.

2.4. Size-exclusion chromatography

The Aβ species either in TBS or 70% FA, obtained as described above, were fractionated on a Superose 12 size-exclusion column (1 cm × 30 cm, Pharmacia Biotech., Uppsala, Sweden) equilibrated with the corresponding mobile phase solution at a flow rate of 0.5 ml/min. Twenty-eight fractions of 1 ml each were collected and analyzed. To determine where Aβ eluted, 100 μl aliquots either from saline-soluble fractions or FA extracts (diluted 1000-fold with 1 M Tris–HCl, pH 8.0) were analyzed by ELISA. For evaluation of lipids, total cholesterol was enzymatically measured using a standard kit (Wako). Under our experimental conditions, plasma lipoproteins were eluted in fractions 7–14 while fractions 15–28 contained cholesterol-free proteins.

2.5. Immunoblot analysis

In order to characterize the distribution of monomeric and oligomeric Aβ eluted on the size-exclusion chromatography, aliquots of 100 μl FA-soluble supernatants or 500 μl saline-soluble supernatants desalted using a 3 kDa cut-off membrane were dried in a rotary vacuum and separated on 10% Tris/Tricine SDS–PAGE using standard protocols. The resulting Aβ species were transblotted onto immobilon P (Millipore) for 45 min at 400 mA using 10 mM CAPS, pH 11, containing 10% methanol [11]. The membranes were blocked with 5% low-fat milk in PBS containing 0.05% Tween 20 and incubated with monoclonal 6E10 1:1000 (anti-Aβ1–16), followed by horseradish peroxidase-labeled sheep anti-mouse $F(ab')_2$ 1:2000 (Amersham). Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit and exposed to Hyperfilm ECL (Amersham).

2.6. Aβ40 and Aβ42 quantitation

Sandwich ELISA [1,32] was used to specifically quantitate whole plasma or LPDP Aβ species, as previously described in detail [20]. Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aβ11–28, specific for Aβ11–16) and sequentially incubated with 100 μl of samples followed by horseradish-peroxidase-conjugated BA27 (anti-Aβ1–40, specific for Aβ40) or BC05 (anti-Aβ35–43, specific for Aβ42 and Aβ43) [1,32]. For the analysis of brain Aβ species, 100 μl of saline-soluble Aβ species were directly subjected to ELISA, whereas the insoluble Aβ samples in the form of 70% formic acid extracts, were neutralized with 1 M Tris–HCl (pH 8.0) and diluted 1:1000 prior to ELISA. The resulting values were corrected with the wet weight of the brain to be finally expressed as pmol/g. The plates were normalized to each other by inclusion of three standard plasma samples on all plates.

2.7. Statistical analysis

Non-parametric methods (Kruskal–Wallis test or Mann–Whitney test) were used. Statistical significance was set at $P < 0.05$. Significant differences among groups were further analyzed using Dunnet's post test for multiple comparisons and correlation studies were made with Spearman's rank correlation. When necessary, a logarithmic transformation was used to achieve a normal distribution for data obtained. All statistical evaluations were performed with the GraphPad Prism, Version 3.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Aβ levels in NC

Spearman's rank analysis of Aβ42 in the normal controls group revealed no statistic correlation with age for either whole plasma (Fig. 1a; $n = 241$) or LPDP (Fig. 1b; $n = 103$) levels of Aβ42 ($P = 0.05$ versus 0.4902) with a mean value ± S.D. of 15.6 ± 2.1 fmol/ml versus 2.0 ± 1.4 fmol/ml, respectively. In relative terms, Aβ42 in lipoprotein-free fraction represented ~3% of total plasma Aβ which remained unmodified until age 50's, followed by a slow but steady decline, 2.1–1.4% between the ages 60 and 100, respectively (Fig. 1c). In contrast, a statistical significant age-dependent increase of Aβ40 over age 70–90's was observed in both whole plasma (Fig. 1d; $n = 241$, $P = 0.0003$) and LPDP (Fig. 1e;$n = 113$; $P = 0.0009$), with a mean value ± S.D. (fmol/ml) as follows—whole plasma: 61.5 ± 32.2 (<60, $n = 172$) versus 103.8 ± 41.5 (>60, $n = 69$); LPDP: 6.7 ± 3.7 (<60, $n = 54$) versus 10.9 ± 6.0 (>60, $n = 59$), respectively. In relative terms, lipoprotein-free Aβ40 represented ~8% of total plasma Aβ, and remained almost unchanged until the age 90 (Fig. 1f). In healthy centenarians, all values tend to slightly fall.

3.2. Aβ levels in AD

In order to identify potential differences between AD patients and age-matched normal controls, the analysis of whole-plasma and lipoprotein-free Aβ species as a function of the degree of cognitive impairment (MMSE scores grouped as >20, 20–11, and <11) was performed. Whole plasma sAβ42 remained slightly elevated in all of three phases of the disease compared with the age-matched normal control group (Fig. 2a), although the differences were not statistically significant ($P > 0.05$). However, as depicted in Fig. 2b, Kruskal–Wallis non-parametric analysis revealed that lipoprotein-free Aβ42 was significantly increased in the initial stages of the disease in comparison with normal control values ($P < 0.01$ for mild AD and $P < 0.001$ for moderate AD, for post hoc comparisons). Mann–Whitney test revealed that Aβ42 levels in LPDP declined with the progression of AD from mild or moderate to severe ($P = 0.018$ for mild and $P = 0.016$ for moderate). In relative terms, the percentage of lipoprotein-free sAβ42 in whole plasma sAβ was only significantly increased in mild AD when compared with age-matched normal controls (Fig. 2c; $P < 0.05$, for post hoc comparisons). The relative percentage was significantly lower in severe AD when compared with mild AD ($P = 0.0316$), but there were no significant differences with the other AD subgroups tested ($P < 0.05$). In the case of sAβ40, the values for whole plasma and lipoprotein-free Aβ40, as well as the relative percentage were similar to normal control values in all stages of the disease, with no statistical differences among the groups tested ($P > 0.05$, Fig. 2d–f).

3.3. Aβ levels in DS

In DS patients, aging appeared to act slightly different in the homeostasis of plasma sAβ levels (Fig. 3). The concentration of whole plasma sAβ42 (Fig. 3a) remained stable until age 40's followed by a significant decrease in the 50's ($P < 0.001$, post hoc comparisons). A similar trend was observed for the LPDP sAβ42. Kruskal–Wallis test revealed a significant decline of lipoprotein-free sAβ42 ($P < 0.001$, post hoc comparisons) in those patients age 40–60's compared with those age 30's or below (Fig. 3b). Almost identical tendency was observed in the age-related change of whole plasma and lipoprotein-free sAβ40 (Fig. 3d and e). In relative terms, the increase of sAβ42 seen in DS at early age may represent a pre-symptomatic AD phase, whereas the subsequent decline may well parallel the disease progression (Fig. 3c and f). This temporal profile obtained in individuals with DS correlate with the values obtained in AD patients with different MMSE scores, although the baseline level appeared to be slightly higher in DS subjects than in AD individuals. Kruskal–Wallis test revealed that in DS teenagers the dissociation of both sAβ42 or sAβ40 from lipoprotein particles is significantly favored (Fig. 3c and f), suggesting that the increase in lipoprotein-free sAβ species

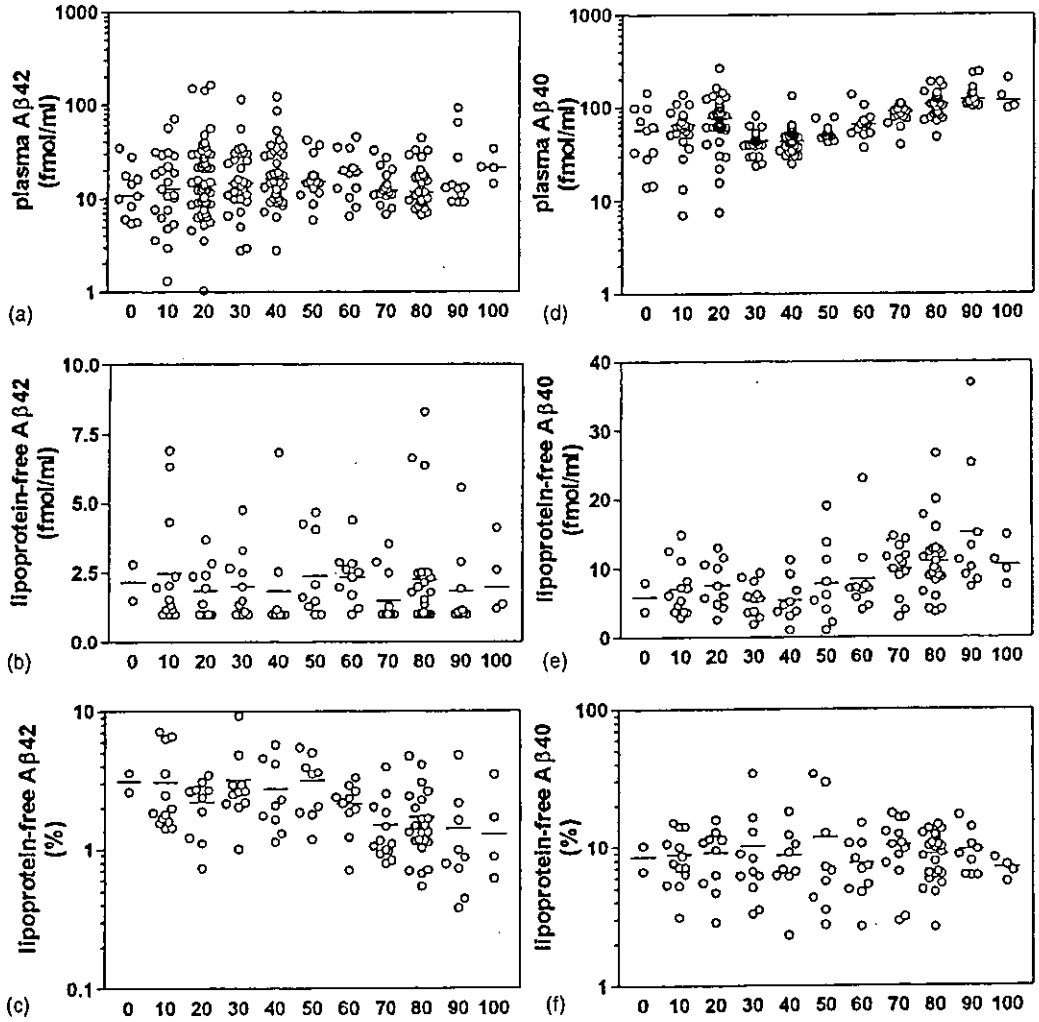

Fig. 1. Age-related changes of plasma and LPDP sAβ species in normal controls. The concentration of sAβ species was measured by captured ELISA, as described in Section 2. (a, d) Quantitation of whole plasma sAβ42 and sAβ40; (b, e) quantitation of lipoprotein-free sAβ42 and sAβ40. The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ are indicated in (c) and (f), respectively. Horizontal bars indicate the median values.

may be associated with the pre-morbidity of metabolic conditions in AD and DS.

3.4. Aβ levels in brain parenchyma

In order to further assess the potential in vivo relevance of lipoprotein-free sAβ in AD pathology, the lipoprotein-associated and lipoprotein-free sAβ species were characterized in cortical brain samples from six AD individuals and four normal controls. Samples were sequentially extracted with TBS pH 7.4 and 70% FA, and the resulting fractions analyzed via size-exclusion chromatography, immunoblot, and ELISA. In all the AD cases tested (upper half of each panel), a different gel-filtration pattern was observed for either saline-soluble or FA-extractable fractions (Fig. 4a and d, upper halves, respectively). Total cholesterol was detected in saline-soluble fractions 7 and 8 as well as in FA-extractable fraction 8. In the TBS samples, monomeric soluble Aβ immunoreactivity (Fig. 4b, upper half) was present in two fractions of different molecular mass: (i) >200 kDa, a fraction enriched in total cholesterol content exhibiting a retention time consistent with the molecular mass of lipoprotein particles (Fig. 4b, upper half, fraction 8), and (ii) 4–8 kDa, fractions containing predominantly monomeric Aβ (fractions 15 and 16) but also detectable levels of dimeric Aβ (fraction 15). ELISA analysis of the saline-soluble Aβ (Fig. 4c, upper half) identified the presence of sAβ40 and sAβ42 in either fraction. The following quantitative values were obtained—fraction 8: 95.14 fmol/ml for Aβ40 and 60.51 fmol/ml for Aβ42; fractions 15 and 16: 375.05 fmol/ml for Aβ40 and 3.07 fmol/ml for Aβ42.

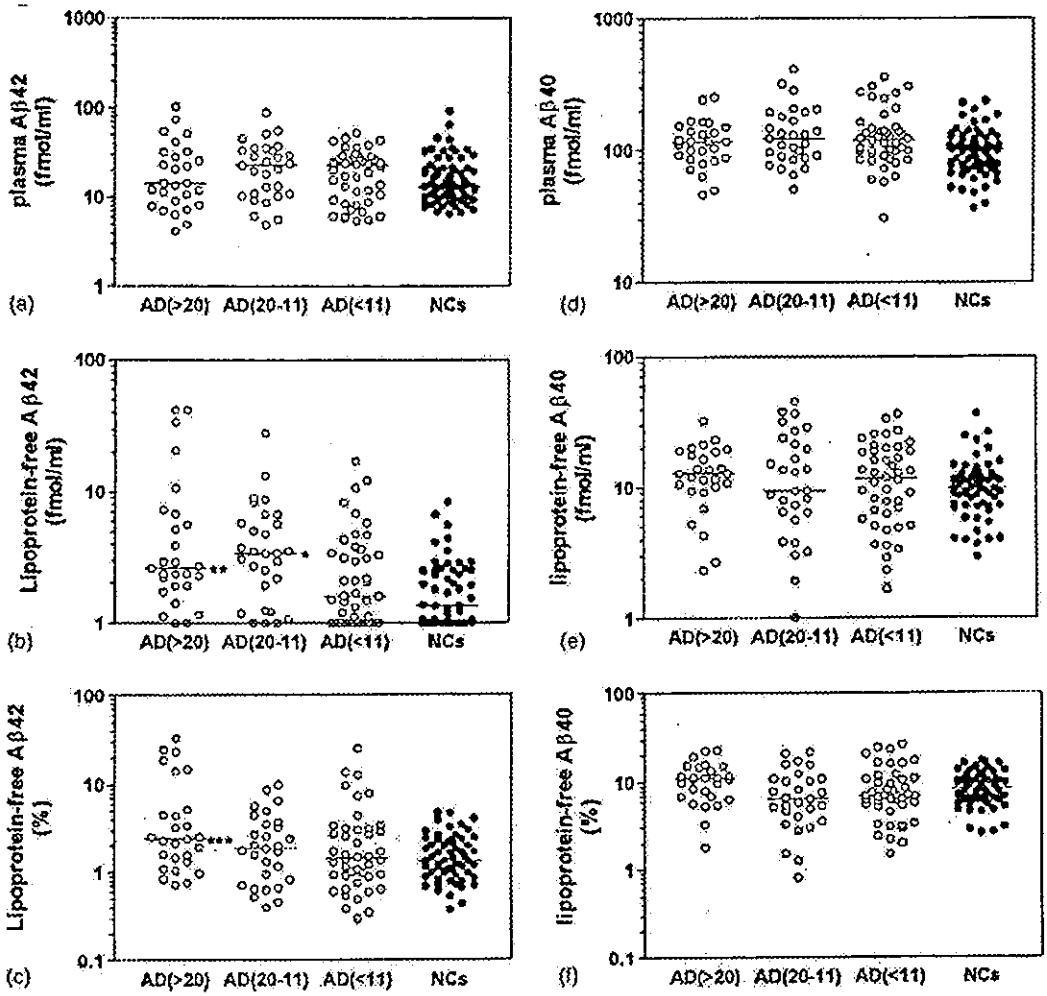

Fig. 2. Variations of plasma and LPDP sAβ species according to the severity of dementia. AD patients (○) were divided into three groups as a function of MMSE; mild AD (MMSE, >21, $n = 27$), moderate AD (MMSE, 11–20, $n = 29$), and severe AD (MMSE, <11, $n = 44$). Quantitation of whole plasma sAβ42 and sAβ40 are indicated in (a) and (d), respectively; lipoprotein-free sAβ42 and sAβ40 are depicted in (b) and (e), respectively. The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ is shown in (c) and (f), respectively. Horizontal bars indicate the median values. The statistical significance compared with age-matched control group (●, NCs; $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$).

Formic acid extractable Aβ isolated from AD brain was identified in several chromatographic fractions. Fig. 4d and e depict a representative elution profile and the corresponding immunoblot analysis employing anti-Aβ monoclonal 6E10. Monomers were identified in fractions 14–15, dimers were present in fractions 12–15 and trimers in fractions 12–14 while larger molecular mass components characteristic of aggregated Aβ species were detected in fractions 11–14. No Aβ immunoreactivity was evident in cholesterol-containing fractions 7–10. Although so much immunoreactivity in fractions 11–14 and less in fraction 15, the ELISA analysis of each fraction revealed the highest Aβ levels in fraction 15, followed by fractions 14 and 16. Consistent with the previous reports showing that the BNT77-based ELISA may not detect oligomeric, SDS non-dissociable Aβ species [2,5], our ELISA failed to capture FA-extractable oligomeric Aβ species, indicating that the values obtained may represent the amount of Aβ monomers.

In all the NC cases tested (lower half of each panel), a similar gel-filtration pattern, although less amount, was observed for either saline-soluble or FA-extractable fractions when compared with AD cases (Fig. 4a and d, lower halves, respectively). Total cholesterol was detected in saline-soluble fractions 7–10 as well as in FA-extractable fraction 8. No Aβ immunoreactivity was detected in either saline-soluble or FA-extractable fractions (Fig. 4b and e, lower halves, respectively). Very little lipoprotein-associated or lipoprotein-free sAβ species was detected in four plaque-free control brains (Fig. 4c, lower half), whereas formic acid extractable Aβ was negligible in any control brains (Fig. 4f, lower half).

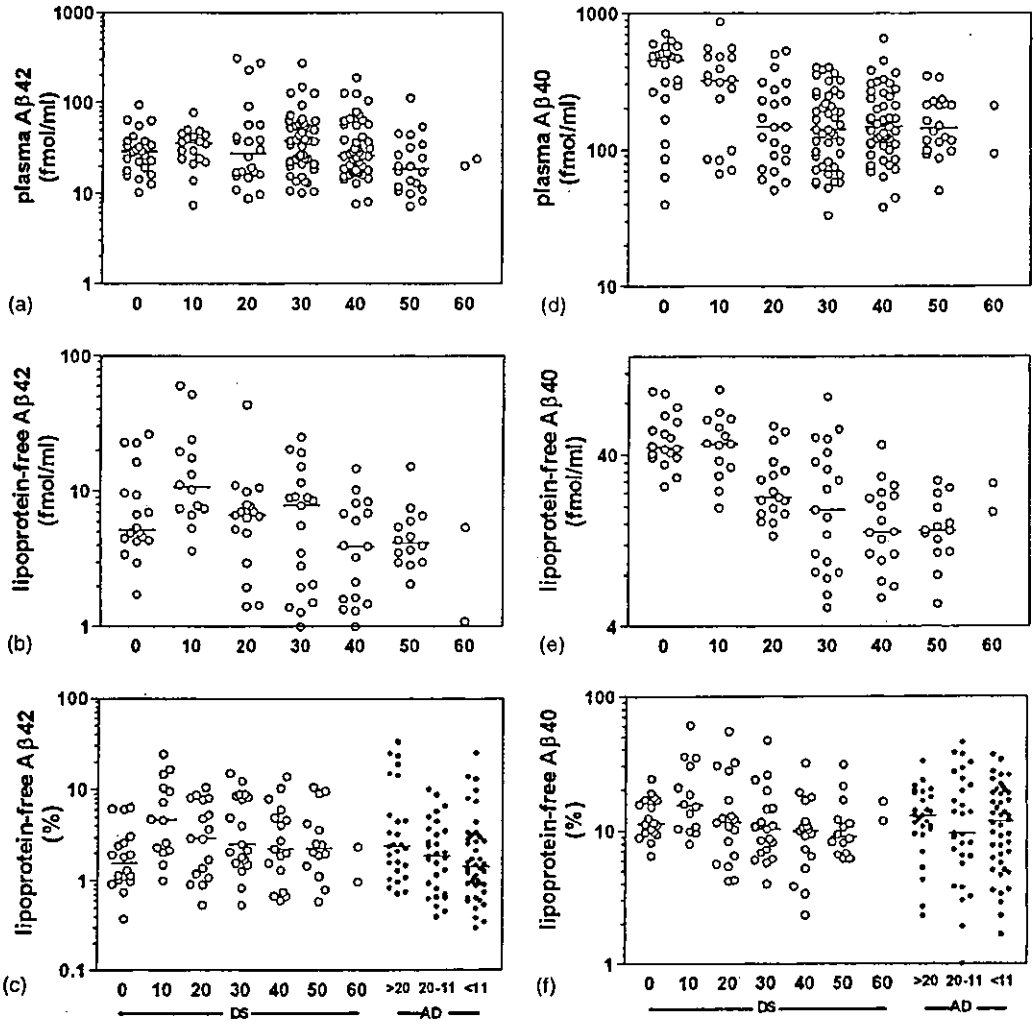

Fig. 3. Age-related changes of plasma and LPDP sAβspecies in Down's syndrome. Quantitation of whole plasma sAβ42 and sAβ40 are indicated in (a) and (d); lipoprotein-free sAβ42 and sAβ40 are shown in (b) and (e). (c, f) The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ in Down's syndrome (○) and Alzheimer's disease (●). AD patients were divided into three groups according to the MMSE, as indicated in Fig. 2.

4. Discussion

Our data suggest that the normal lipoprotein–sAβ40 interaction in plasma is under more strict control than sAβ42 throughout the entire human life span. While Aβ40 values remain almost constant, the lipoprotein–sAβ42 interaction appeared to be reshaped in normal controls over-60's. The values obtained in sporadic AD patients remained increased in comparison with age-matched controls over-60's, indicating that sporadic AD patients, particularly mild AD, are less protected by lipoproteins. Due to the well known association of sAβ with HDL particles [14] and its low excretion into urine [8], it is conceivable that sAβ catabolic/excretory pathway(s) may follow those of lipoprotein particles. In this sense, functional decline of lipoprotein particles to reshape sAβ metabolism may provide not only the metabolic conditions to initiate and/or accelerate the cascade favoring Aβ oligomerization, but also result in reduced clearance of amyloidogenic lipoprotein-free sAβ peptides from the brain. Inadequate clearance of amyloidogenic lipoprotein-free Aβ may vary among AD and/or DS patients, resulting in the fluctuated values found in plasma studied.

It has been reported that abundant diffuse Aβ42 plaques are already present in teenage DS subjects [31]; almost every patient, aged 30 years and older, further develop Congo-red positive senile plaques [17,37]. In order to conduct further investigations, we selected to study DS patients whose brains at different ages represent pre-AD and various stages of the AD pathology. Our data suggests that in DS patients, changes in metabolic conditions favor the dissociation of sAβ from its lipidic environment during early age, resulting

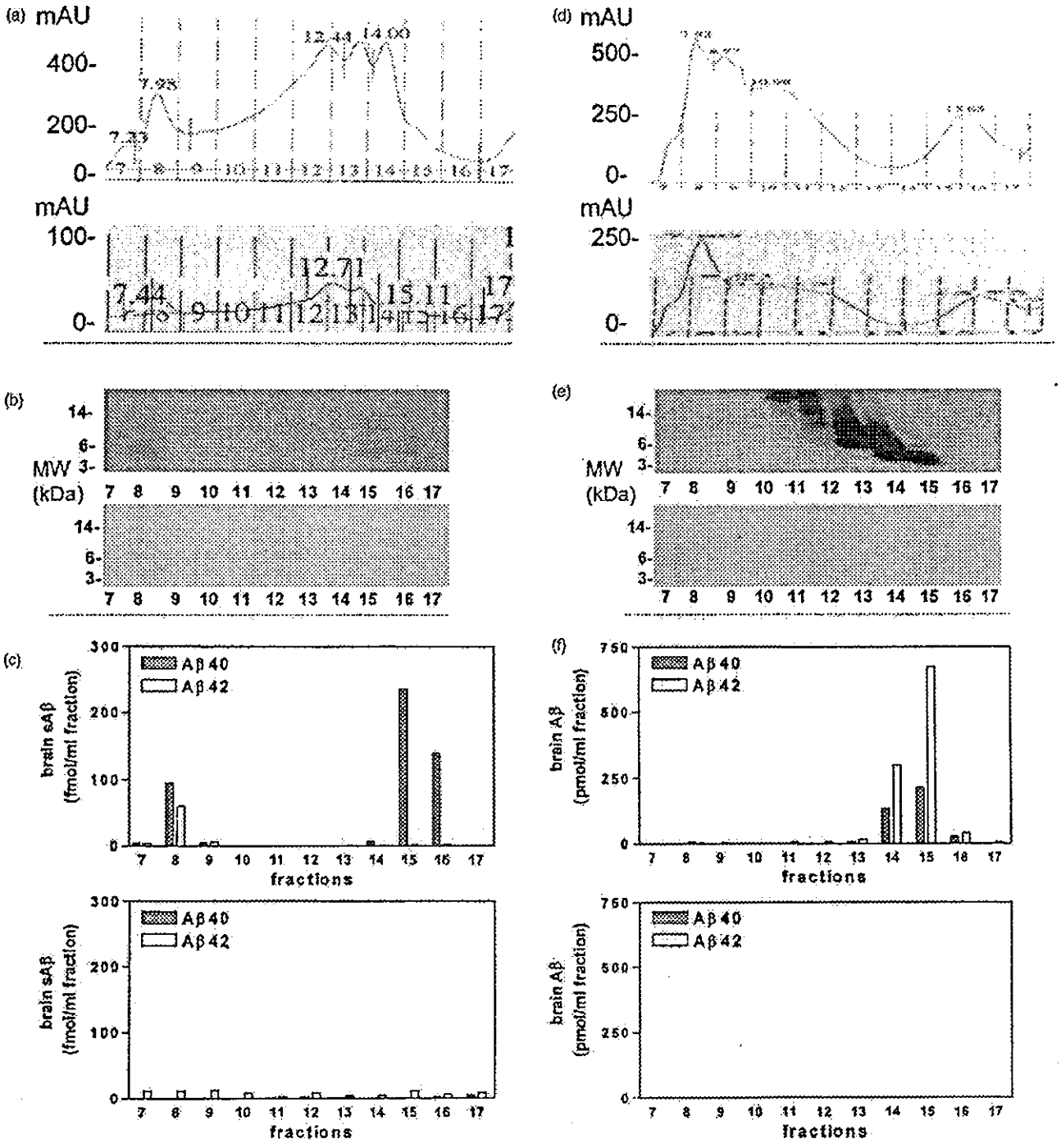

Fig. 4. Characterization of brain soluble Aβ/Aβ species in Alzheimer's disease and nonnal control individual. Elution profiles of saline-soluble and saline-insoluble, formic acid extractable Aβ are indicated in (a) and (d). Immunodetection of eluted Aβ species are shown in (b) and (e). (c, f) The presence of Aβ42 and Aβ40 in the different fractions. All upper half of panels are a representative result of extractions carried out in six AD cases. All lower half of panels are a representative result of extractions carried out in four NC cases.

in increased levels of amyloidogenic lipoprotein-free sAβ species, both Aβ40 and Aβ42, in plasma. Free-sAβ values decrease as the disease progresses, likely mirroring the on-going AD pathology in the DS brains. This is in agreement with our published data [12] indicating that highly significant decrease of plasma and CSF Aβ are linked to the marked deposition of Aβ in Tg2576 mice brain.

Kuo et al. isolated and quantitated brain sAβ via ultracentrifugation and molecular sieving [15] and found a continuous distribution of monomeric and oligomeric

sAβ ranging from <10 to >100kDa. It is possible that the former represents lipoprotein-free sAβ and the latter, lipoprotein-associated sAβ species. Fagan et al. [3] reported that this dissociation also occurs in the CSF where sAβ is also associated with lipoprotein particles [13]. Our experiments using 10 brains (six AD and four NCs) correlate with those results; both sAβ species were successfully eluted from a gel-filtration matrix, suggesting that the dissociation of sAβ from lipoprotein particles occurs in brain parenchyma. It is not known whether brain specific chaperones further modulate the conversion of lipoprotein-free sAβ into amyloid fibrils via oligomeric intermediates. However, it is relevant to note that lipoprotein-free brain sAβ forms native dimers in AD brains, a feature consistently found in the six AD brains processed. The presence of less soluble free-sAβ versus much more Aβ42 amyloid in AD brains suggests that the former specie could be highly amyloidogenic in vivo, resulting in the fast conversion from the former to the latter. Alternatively, it can be speculated that our BNT77-based ELISA specific for Aβ monomer failed to detect the presence of soluble dimeric sAβ42. As discussed by several investigators, soluble Aβ oligomers appear to be the pathological amyloidogenic molecule [9–11,16,25–27,38,39]. The present data support our hypothesis that the dissociation of sAβ from or the lack of association with lipoprotein particles constitutes a potential mechanism to initiate and/or accelerate the cascade favoring Aβ oligomerization in brain. In this regard, the presence of dimeric lipoprotein-free sAβ in AD patients but not in NCs suggests that lipoproteins are not innocent bystanders but rather major determinants to balance sAβ homeostasis in biological fluids and brain parenchyma.

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特集：痴呆の早期診断・治療と総合的機能評価—もの忘れ外来の現状と役割—

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1．中核症状に対する薬物療法

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特集

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はじめに

本邦で現在市販されている痴呆症の中核症状に有効な薬剤は，アルツハイマー型痴呆(以下，AD)に対する塩酸ドネペジルのみである．塩酸ドネペジルは，単に認知機能の改善だけではなくQOLの改善をもたらし，多くの恩恵を与えている．今後，より有効な薬剤が市販されると考えられるが，現時点ではこの塩酸ドネペジルを効果的に使うことができるかが，われわれ臨床家に問われるところである．

そこで本稿では，塩酸ドネペジルの効果，使い方，注意点，AD以外の痴呆への効果などを紹介し，今後の展望について述べる．

ADに対する塩酸ドネペジルの効果

塩酸ドネペジルは，ADの脳内で減少したアセチルコリン(Ach)を増やすことによって，記憶を改善する対症療法薬と位置づけられる．

自験例での有効性をまとめると，対象43例中49%(21例)に改善がみられ，不変が35%(15例)，悪化７%(３例)，中止９%(４例)であった[1]．この結果は，国内におけるその他の報告とも一致している[2]．改善例の中には，行きつけの店へも買い物に行けなくなった74歳の女性が，塩酸ドネペジル内服により，忘れずに覚えていることが多くなっただけではなく，幼稚園の先生をしている娘さんの仕事の手伝いをきちんとできるようになった著効例もある．

また現在，塩酸ドネペジルは軽度から中等度のADが適応となっているが，重症例でも有効例がある．われわれは，会話がほとんどかみ合わなくなった重症例で，塩酸ドネペジルの投与により意欲的となって会話の内容もかみ合うようになり，さらに絵を描けるようになった症例を経験した．最初は色を塗りつぶすだけであったが，次第に線が書け，次いで丸が書けるようになり，形を成すようになった．その後，３年を経過した現在も絵を続けて描いていて，しかもクレヨンから絵の具へと，使う道具にも進歩がみられている[3]．

ADは進行性の病気であり，“不変”の考え方が重要である．例えば腹痛など通常の病気であれば，不変は改善していないことになるが，ADでは不変イコール進行抑制と考えることができる．また，図１のように，塩酸ドネペジル投与後，約１年経過すると徐々に悪化してくるといわれているが，全例がそうなるわけではなく，良好な状態が維持される症例もある．そういう点からも，塩酸ドネペジルによる症状の進行抑制はQOLの維持，通院加療期間の延長などにつながり，医療経済学的にみても非常に有用である．

軽度認知障害(MCI)に対する塩酸ドネペジルの効果

ADの前段階として，MCI(mild cognitive impairment)という概念が提唱されている．Petersenら[4]が提唱したMCIの定義は，①自覚的なもの忘れの訴えがある，②客観的な記憶障害を認める，③記憶障害以外の

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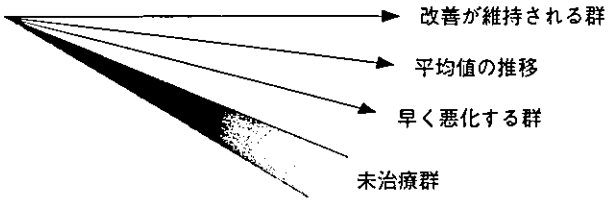

図1　塩酸ドネペジルの長期経過

高次機能障害がない，④日常生活動作は保たれている，⑤痴呆の診断基準を満たさない，というものである．このMCIの定義には現在のところ一致した見解が得られていないが，少なくとも正常とADの間に移行期のような状態が存在することは確かであり，痴呆症の前段階あるいは極めて早期のADをとらえている可能性がある．

わが国ではMCIに対する塩酸ドネペジルの適応はないが，自験例で「もの忘れが改善した」あるいは「頭がスッキリした」という自覚が得られ，長谷川式簡易知的機能検査-改訂版(HDS-R：Hasegawa's dementia scale-revised)あるいはMMSEなどのスコアの改善もみられた症例を経験した．

欧米では塩酸ドネペジルをはじめ，各種薬剤のMCIに対する臨床試験が行われている．米国でのMCI患者270例を対象とした多施設共同二重盲検プラセボ対照比較試験では，プラセボ投与群に比し塩酸ドネペジル投与群で24週後のmodified ADAS-cog total scoreが有意に改善することが示された(図2)[5]．また，患者の全般評価においても悪化例はプラセボ群に多く，塩酸ドネペジル投与群では改善例が多いという結果が得られている．

Very mild ADに対する塩酸ドネペジルの効果

Very mild ADを対象とした多施設臨床試験が最近米国でなされ，大変興味ある結果が得られた．153例のvery mild ADを対象として，塩酸ドネペジル10 mg/日で24週間投与する多施設共同二重盲検プラセボ対照比較試験が施行された．対象の選定基準としては，CDR (clinical dementia rating)0.5～1.0で，MMSE(mini mental state examination)は21～26点とし，有効性の評価はmodified ADAS(Alzheimer's disease assessment scale)-cogとMMSEを用いている．その結果，modified ADAS-cogのtotal score，MMSEともに塩酸ドネペジル投与群でプラセボ群と比較して有意な改善

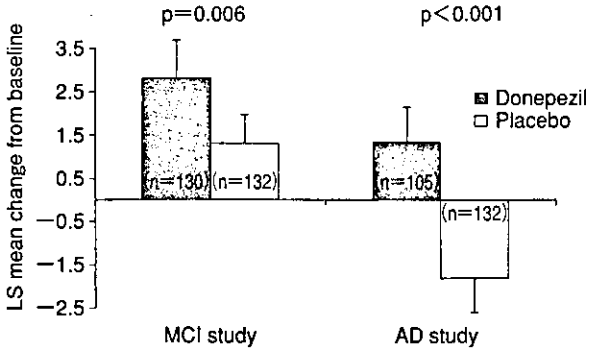

図2　MCIとADを対象とした塩酸ドネペジルの24週目のmodified ADAS-cog total score

がみられた．最も興味深いのは，図3に示すごとくmodified ADAS-cogのcognitive performanceにおいて，特にvery mild AD群で最も良い改善効果を示したことである．また，MMSEでも同様の結果を示した．塩酸ドネペジルをADのより早期から投与する意義が証明されたものと考えられる．

AD以外の痴呆への効果

レビー小体型痴呆(DLB)では，AD同様にアセチルコリン系神経系が障害されており，このため塩酸ドネペジルが有効と考えられている[6]．脳血管性痴呆(VD)では，欧米で二重盲検比較試験が既に行われており，有意な改善効果が報告されている[7]．統合失調症[8]やダウン症候群[9]の認知機能低下にも改善効果がみられたとする報告もなされている．

期待される根本治療薬と塩酸ドネペジルの将来的意義

近年，ADの治療薬開発は，根本的な治療を目指した研究が世界的規模で極めて精力的に行われている．現在最も先端を行っているのは，βおよびγセクレターゼ阻害薬とアミロイドβ蛋白ワクチン療法[10]などである．どちらもADの最も早期病変と考えられるアミロイドβ蛋白(Aβ)の沈着を防ぐ，あるいは消去する治療的アプローチである．詳細は本誌別稿で述べられるので，そちらを参照されたい．このような根本治療薬開発がなされてきている中で，塩酸ドネペジルの将来的意義としては，①来るべき根本治療薬への重要なリリーフ役，②対症療法薬として今後も重要な役割をもつ，の2つがあると考えられる．①については塩

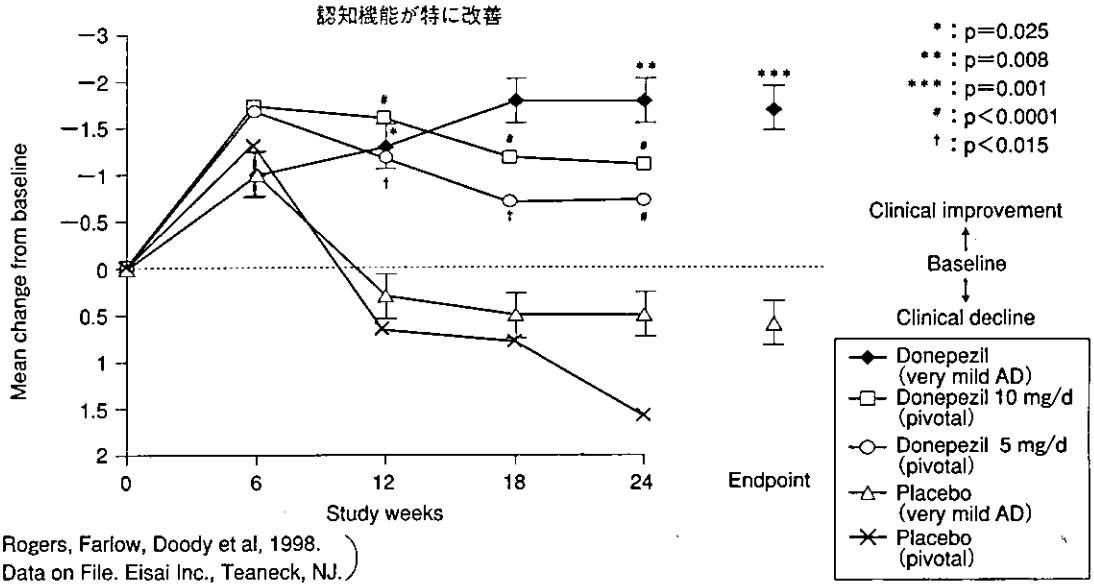

図3　Modified ADAS-cog total score

酸ドネペジルの，症状の進行抑制効果であるが，少しでも進行を防ぐことができれば，来るべき根本治療薬に間に合う可能性が出てくるということである．②については，根本治療薬ができても対症療法薬が不要になることはないということである．神経内科領域では，重症筋無力症という病気があり，既に胸腺摘出術やステロイド療法といった根本療法が確立されているが，対症療法であるAchE阻害薬は不要になっていない．実際，この対症療法薬であるAchE阻害薬を投与したときが患者にとって筋力回復を自覚でき，最も喜ばれるのである．このような事実からも，対症療法薬である塩酸ドネペジルは今後も重要な役割を担っていくと考えられる．

今後の検討課題

ADの治療では，薬物療法だけではなく非薬物療法との併用が有効である可能性がある[11]．そのような観点から，様々な非薬物療法が試みられており，われわれもアロマセラピーについて検討した．その結果，軽度から中等度のAD患者において，自発性および感情機能のみならず知的機能にも改善傾向が示された．今後は，さらに多数例で検討していきたいと考えている．非薬物療法的介入の薬物療法との併用効果について明らかにしていくことも大切である[12]．

前述のように，根本治療薬の開発が進んでいるが，対症療法は根本治療が可能になったとしてもいつでも必要なものであり，塩酸ドネペジルはリリーフ役としても重要な役割を担っている．しかし，現時点ではADに対する効果に関して，反応が良好な群(responder)と良好でない群(non-responder)の存在が知られており，その差異の解明が大きな課題となっている．われわれはAch受容体(AchR)に着目し，AchRα7の遺伝子多型の検討によりnon-responder群に比しresponder群で，ヘテロの頻度が有意に多いことを明らかにした[13]．まだ例数が少なくさらなる検討が必要であるが，AchRα7遺伝子多型の検査が塩酸ドネペジルの有効性の予知に役立つ可能性が示唆される．今後，真のresponderとnon-responderを区別するパラメータの解明が必要である．

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要　旨

現在のところ，わが国で市販されているアルツハイマー型痴呆(以下，AD)治療薬は塩酸ドネペジルのみである．塩酸ドネペジルは対症療法薬ではあるが，単に認知機能の改善だけではなくQOLの改善をもたらし，多くの恩恵を与えている．このための，より早期の段階で治療する試みがなされてきている．欧米でMCIやvery early ADを対象として治験がなされているが，どちらも有効性を示す結果が得られている．近年，ADの治療薬開発は，根本的な治療を目指した研究が世界的規模で極めて精力的に行われている．このような根本治療薬開発がなされてきている中で，塩酸ドネペジルの将来的意義としては，①来るべき根本治療薬への重要なリリーフ役，②対症療法薬として今後も重要な役割をもつ，の2つがあると考えられる．今後の課題の1つとして，ADに対する効果に関して，反応が良好な群(responder)と良好でない群(non-responder)の差異の解明が求められている．われわれは，アセチルコリン受容体(AchR) α7の遺伝子多型の検討により，non-responder群に比しresponder群でヘテロの頻度が有意に多いことを明らかにし，AchRα7遺伝子多型の検査が塩酸ドネペジルの有効性の予知に役立つ可能性を指摘した．今後，responderとnon-responderを区別するパラメータのさらなる解明が必要である．

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Brain-derived neurotrophic factor gene polymorphisms and Alzheimer's disease

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Summary. Several lines of evidence have made brain-derived neurotrophic factor (BDNF) an important candidate gene conferring risk for Alzheimer's disease (AD). Recently, three studies reported an association between two single-nucleotide polymorphisms (SNP) – i.e., C270T and G196A – in the BDNF gene and AD. This attempt to confirm these associations in a larger AD sample included examination of the linkage disequilibrium of these two SNPs. Comparison of 487 Japanese AD subjects with 471 cognitively normal elderly controls showed higher frequencies of the G allele (60.5 vs. 55.5%, $p=0.028$) and of both the GG and GA genotypes (85.8 vs. 79.8%, $p=0.025$) of the G196A polymorphism in AD subjects than in controls and higher frequency of the T allele of the C270T polymorphism in AD subjects who were negative for apolipotrotein E4 (2.0 vs. 4.4%, $p=0.035$) or positive for AD family history (2.8 vs. 7.1%, $p=0.046$). These findings suggest that BDNF gene polymorphisms play some role in the development of AD.

Keywords: Alzheimer disease, apolipoprotein E (ApoE4), brain-derived neurotrophic factor, case-control study, genetic association, single nucleotide polymorphism (SNP).

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by loss and atrophy of basal forebrain cholinergic neurons and the limbic structures (Terry, 1994). The genetics of AD is complex, and mutations of the genes encoding presenilin-1 (Sherrington et al., 1995), presenilin-2

(Levy-Lahad et al., 1995), and amyloid precursor protein cause the relatively rare, early-onset, autosomal dominant familial form of AD (Goate et al., 1991). The ε4 allele of the apolipoprotein (ApoE) gene (ApoE4) is the major known genetic risk factor for late-onset, sporadic AD (Saunders et al., 1993). However, because these genetic markers cannot explain the overall genetic susceptibility, it is clear that additional genes are involved in the development of AD.

Brain-derived neurotrophic factor (BDNF) protects cholinergic neurons of the basal forebrain and neurons in the hippocampus from ischemia-induced neuronal cell death (Pringle et al., 1996). Reduced mRNA expression of the BDNF protein has been observed post mortem in the parietal cortex of patients with AD (Holsinger et al., 2000), and lower levels of BDNF protein have been reported in the entorhinal cortex (Narisawa-Saito et al., 1996) and in the hippocampus and parietal cortex (Hock et al., 2000). Although another study reported increased BDNF level in the AD brain (Durany et al., 2000), the conflict in these lines of evidence suggest the need to investigate the BDNF gene as an important candidate for AD development.

To our knowledge, only three studies have examined the association between BDNF gene polymorphisms and AD (Kunugi et al., 2001; Riemenschneider et al., 2002; Ventriglia et al., 2002). Two of them found a significant association between the T allele of the C270T polymorphism in the non-coding region of BDNF gene and AD (Kunugi et al., 2001; Riemenschneider et al., 2002). The third, examining an association between G196A (val66met) polymorphism (dbSNP number rs6265) and AD, showed overrepresentation of the GG genotype in AD, independent of ApoE4 status (Ventriglia et al., 2002). The G196A polymorphism is located in the 5′ BDNF precursor peptide (proBDNF) sequence that is proteolytically cleaved to form the mature protein posttranslationally (Seidah et al., 1996).

This study was designed to examine the associations between the C270T and G196A BDNF gene polymorphisms and AD in a larger sample than had previously been studied, as well as to examine the linkage disequilibrium between the two polymorphisms. To eliminate the possibility of racial differences and address the highly heterogeneous etiology of AD, we limited this case-control study to Japanese subjects.

Material and methods

The Ethics Committee of the National Institute on Alcoholism, Kurihama National Hospital (now National Hospital Organization, Kurihama Alcoholism Center) approved this study, and all participants or their families gave informed consent.

Subjects

The AD group consisted of 487 Japanese patients (147 males and 340 females; mean age 76.1 ± 8.9 years) who met National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for "probable" AD (McKhann et al., 1984). The age at onset of obvious cognitive dysfunction, including memory problems, was obtained from spouses or relatives and served to identify the age at onset of AD. Among the AD cases, 101 (32 males and 69 females)