observe any binding of the AR, acetylhistone H3, or p300 to the distal ARE site in the Gas6 gene (Fig. 5*F*).

DISCUSSION

The effect of testosterone replacement therapy on atherosclerosis is controversial (21–25), although testosterone deficiency is known to be associated with cardiovascular disease in men (26–30). We and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function (27), increased carotid intima-media thickness (28), and aortic calcification (9). Recently, testosterone has also been reported to inhibit VSMC proliferation and neointima formation (7), suggesting a direct action of testosterone on the vasculature. In this *in vitro* study we examined the effect of androgens on P_i-induced VSMC calcification and found that androgens at physiological concentrations exhibited inhibitory effects on VSMC calcification. In contrast to the present study, it has been reported that androgens induced vascular calcification in apolipoprotein E knockout mice (31). This discrepancy may derive from the complex *in vivo* effects of testosterone. Further work is required to define the role of androgens in vascular calcification.

Androgens act mainly through transcriptional control of target genes mediated by the nuclear AR (11, 32). In the present study we found that the AR was expressed predominantly in the nucleus of VSMC and had transcriptional activity. Recently, it was demonstrated that the AR-dependent action of androgens protects against angiotensin II-induced vascular remodeling (33). Consistent with this, our results showed that the inhibitory effect of androgens on VSMC calcification was mediated by the AR and not by estrogen receptor.

Recently, we demonstrated that apoptosis plays a central role in the process of P_i-induced VSMC calcification through down-regulation of the Gas6-mediated survival pathway (16, 17). In the present study we found that androgens prevented VSMC apoptosis and restored Gas6 expression and Akt survival signaling. These inhibitory effects of androgens on apoptosis and calcification were eliminated by flutamide and Gas6 siRNA. Our findings indicate that AR-dependent restoration of Gas6 by androgens contributes to the inhibition of apoptosis and VSMC calcification.

Although the involvement of other molecules such as protein kinase Cδ (7) and endothelial nitric-oxide synthase (33) in the vasoprotective actions of androgens is unclear, our data showed that Gas6 plays a pivotal role in the inhibitory effect of androgen on P_i-induced calcification. Several genes containing AREs and having AR-mediated actions have been identified (34, 35). However, little is known about transcriptional regulation and the target genes of the actions of the AR in the vascular system. In this study we identified two AREs in the promoter region of the Gas6 gene and characterized specific direct binding of the AR to the proximal ARE, in contrast to the nonfunctional distal ARE. Interestingly, Mo *et al.* (36) identified that an estrogen response (ER) element spanning −72 to −89 bp from the translation start site in Gas6 and ERα is recruited by estrogen-mediated stimulation of Gas6 gene expression in mouse mammary epithelial cells. In the human Gas6 promoter domain, we also found the existence of an estrogen response element at −243 to −251 bp. In clinical studies, a low serum estradiol level in women was correlated with increased arterial calcification (37), and estrogen replacement could reduce coronary calcification (38, 39). However, in experimental studies, estradiol treatment showed variable effects on vascular calcification with either inhibition (40, 41) or stimulation of calcification (42). Further studies are needed to elucidate the actions of estrogens in vascular calcification.

In summary, this study showed that Gas6 is a novel target that is directly and transcriptionally regulated by the AR, and direct interaction of the AR and Gas6 mediates the inhibitory effects of androgens on vascular calcification. This study provides a new mechanistic insight into the vascular protective action of androgens.

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Low testosterone level as a predictor of cardiovascular events in Japanese men with coronary risk factors

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Objective: Recent epidemiological studies have found that testosterone deficiency is associated with higher mortality largely due to cardiovascular (CV) disease in community-dwelling older men. We investigated whether a low plasma testosterone level could predict cardiovascular events in middle-aged Japanese men with coronary risk factors.

Methods: One hundred and seventy-one male outpatients (30–69 years old, mean ± SD = 48 ± 13 years) who had any coronary risk factor (hypertension, diabetes, dyslipidemia, smoking, and obesity) without a previous history of CV disease were followed up. At baseline, the subjects underwent examination of coronary risk factors, measurement of flow-mediated dilation (FMD) of the brachial artery as an indicator of vascular endothelial function and assays of plasma total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol.

Results: During the mean follow-up period of 77 months, a total of 20 CV events occurred. Kaplan–Meier survival analysis by tertile of plasma hormone levels revealed that the subjects with the lowest testosterone tertile were more likely to develop CV events than those with the highest tertile ($P < 0.01$ by log-rank test). Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone (<14.2 nmol/L) had an approximately 4-fold higher CV event risk compared to those with the higher testosterone tertiles after adjustment for coronary risk factors including medication and FMD (unadjusted hazard ratio, 3.61; 95% CI, 1.47–8.86: multivariate-adjusted hazard ratio, 4.61; 95% CI, 1.02–21.04). Multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

Conclusions: A low plasma testosterone level is associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This is the first report to show the relationship between endogenous testosterone and CV events in Asian population.

1. Introduction

Plasma testosterone level declines with advancing age in men [1]. Testosterone deficiency is often associated with age-related diseases such as erectile dysfunction, osteoporosis, depressed mood, cognitive impairment and frailty [2,3]. Furthermore, a number of studies suggest that testosterone deficiency is related to cardiovascular (CV) disease and its risk factors in men. Inverse relations between testosterone level and coronary risk factors including obesity [4,5], hypertension [5,6], dyslipidemia [4,5], and diabetes [5,7] have been reported. In addition, we and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function [8], increased carotid intima-media thickness [9] and aortic calcification [4]. Although these data do not indicate a causal relationship between endogenous testosterone and CV disease, recent epidemiological studies have demonstrated that community-dwelling older men with a low testosterone level are more likely to die [10–12], largely due to CV disease [11,12]. However, this issue remains unknown in Asian population.

Based on these backgrounds, we tested the hypothesis that a low testosterone level is an independent risk factor for CV disease even in middle-aged Japanese men with coronary risk factors. For this purpose, we conducted a survey of 171 male patients by using baseline clinical information and by measuring sex hormone levels in stored plasma.

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2. Methods

2.1. Subjects

Male subjects aged 30–69 years at baseline, who were referred to our department to check for CV disease and undergo examination of vasomotor function of the brachial artery in 1996–2000, and had any of the classical coronary risk factors including hypertension, dyslipidemia, diabetes mellitus and current smoking, were eligible. Hypertension, dyslipidemia and diabetes mellitus were defined according to diagnostic criteria [13–15] or if the subject was taking any medication for these diseases. Subjects with a history of CV disease, including stroke, coronary heart disease, congestive heart failure and peripheral arterial disease, were excluded. Malignancy, overt endocrine disease and use of steroid hormones were also excluded, because these conditions may have a significant influence on both plasma sex hormones and clinical course.

Of the 188 eligible subjects whose plasma was stored, written informed consent was obtained from 171 subjects; 1 subject refused and 16 subjects were lost to follow-up. Then, plasma hormone levels were measured and follow-up data were obtained in 171 subjects. The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo. Each subject or a family member, if the subject had died, gave written informed consent for enrollment in this study.

2.2. Clinical measurements

Clinical information was collected at baseline when each patient attended our department. Blood sampling and measurement of height, weight, blood pressure and vasomotor function were performed in the morning after a 14-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare Co., Ltd., Kyoto, Japan) on the nondominant arm in a sitting position, and the average was used for analysis.

Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol concentration was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Plasma glucose concentration was assayed by the glucose oxidase method, and hemoglobin A1c level was measured by high-performance liquid chromatography.

Plasma concentrations of total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol were determined using sensitive radioimmunoassays by a commercial laboratory (SRL, Inc., Tokyo, Japan). Because the plasma used for hormone assays was deep-frozen (−80 °C) for up to 7 years, we checked the change in titers using the stored samples, which had been measured at sampling 5–7 years before. Pearson's correlation coefficient between the two measurements was 0.965 for estradiol ($n = 34$), 0.976 for testosterone ($n = 20$), 0.991 for DHEA-S ($n = 15$) and 0.937 for cortisol ($n = 16$), indicating that there was no significant change in plasma titers in our frozen samples. The intra-assay coefficients of variation for the measurements were less than 5%.

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously [16]. Briefly, endothelium-dependent flow-mediated vasodilation (%FMD) was measured as the maximal percent change in the vessel diameter after reactive hyperemia. Subsequently, endothelium-independent nitroglycerin-induced vasodilation was measured as the maximal percent change in the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo). The same examiner (M.H.) performed the measurements of FMD throughout this study.

2.3. Follow-up

The subjects were followed in 2006–2007 by mail and/or visits to our clinic. Each subject or a family member completed the questionnaire on CV disease and health status. CV events analyzed as the endpoints of this study included stroke, coronary artery disease, sudden cardiac death, and peripheral arterial disease. If CV events were reported on the questionnaire, we attempted to confirm the diagnosis of each event by medical records and/or interview by research doctors who were unaware of the patient's plasma hormone levels. Finally, after thorough examination, 20 cases were determined as CV events. Eighteen cases were ascertained by medical records which included clinical course, physical examination, laboratory tests and imagings. Because medical records were not available on other two cases of self-reported ischemic stroke, they were diagnosed according to the phone interview to each patient.

2.4. Data analysis

Values are expressed as mean ± SD in the text unless otherwise stated. Differences between the groups were analyzed using ANOVA for continuous variables and Chi-squared test for categorical variables. Survival was analyzed using Kaplan–Meier plots and log-rank tests. Hazard ratios (HRs) for CV events were analyzed using Cox proportional hazards regression. A value of $P < 0.05$ was considered statistically significant. Data were analyzed using SPSS (Ver. 17.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of subjects according to plasma testosterone level

Table 1 shows the baseline characteristics of the subjects by tertile of plasma testosterone. As reported previously [4–8], subjects with the lowest testosterone tertile tended to be obese, hypertensive, dyslipidemic, diabetic, and to have impaired endothelial vasomotor function compared to those with higher testosterone tertiles. Age and smoking status were not different between the groups.

3.2. CV events and hormones

During the mean follow-up period of 77 ± 46 months (median = 54 months), a total of 20 CV events occurred (Table 2). Eleven cases of coronary artery disease included three of myocardial infarction, three of medically treated angina pectoris, four of percutaneous coronary intervention, and one of coronary artery bypass grafting. All of the five cases of stroke were due to cerebral infarction.

As shown in Fig. 1, Kaplan–Meier survival analysis by tertile of plasma testosterone level revealed that low testosterone was associated with CV events. Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone, but not those with the middle tertile, had significantly increased risk for CV events compared to those with the highest tertile (Table 2). Adjustment for age and body mass index did not attenuate the effect.

Then, HRs for the lowest tertile of plasma testosterone vs. the higher (middle and highest) tertiles were analyzed. The subjects with the lowest tertile (<14.2 nmol/L) showed an unadjusted HR of 3.61 (95% CI, 1.47–8.86), and an adjusted HR of 4.24 (95% CI, 1.67–10.78) for age, body mass index, and current smoking. The HR was 4.61 (95% CI, 1.02–21.04) after adjustment for age, body mass index, current smoking, systolic blood pressure, HDL cholesterol, non-HDL cholesterol, hemoglobin A1c, %FMD,

Table 1
Baseline characteristics of subjects by tertile group of plasma testosterone.

	Tertile 1 <14.2 nmol/L (*n* = 57)	Tertile 2 14.2–19.4 nmol/L (*n* = 57)	Tertile 3 >19.4 nmol/L (*n* = 57)	*p* for trend
Testosterone (nmol/L)	11.0 ± 3.0	17.0 ± 1.6	24.0 ± 3.0	<0.001
(ng/dL)	(318 ± 86)	(490 ± 45)	(693 ± 86)	
DHEA-S (μmol/L)	4.94 ± 2.68	4.55 ± 2.25	4.83 ± 2.64	0.81
Estradiol (pmol/L)	115 ± 30	116 ± 31	133 ± 30	0.004
Cortisol (nmol/L)	386 ± 138	378 ± 142	361 ± 120	0.67
Age (years)	47 ± 13	45 ± 13	50 ± 14	0.24
Body mass index (kg/m^2)	27.6 ± 5.5	25.6 ± 4.3	24.1 ± 3.6	<0.001
Systolic blood pressure (mmHg)	131 ± 18	125 ± 16	123 ± 12	0.01
Diastolic blood pressure (mmHg)	79 ± 15	74 ± 11	74 ± 9	0.04
Non-HDL cholesterol (mmol/L)	4.19 ± 1.27	3.91 ± 1.06	3.74 ± 1.01	0.10
HDL cholesterol (mmol/L)	1.20 ± 0.36	1.23 ± 0.41	1.44 ± 0.48	0.005
Triglycerides (mmol/L)	2.04 ± 2.12	1.91 ± 1.85	1.46 ± 1.28	0.18
Fasting plasma glucose (mmol/L)	6.00 ± 1.18	5.73 ± 0.92	5.73 ± 1.28	0.34
Hemoglobin A1c (%)	5.9 ± 1.7	5.2 ± 0.8	5.5 ± 1.2	0.03
%FMD	4.2 ± 2.7	5.7 ± 4.2	6.1 ± 3.8	0.01
%NTG	12.8 ± 4.3	14.2 ± 5.4	13.2 ± 5.0	0.30
Hypertension, *n* (%)	30 (53)	20 (35)	20 (35)	0.09
Dyslipidemia, *n* (%)	33 (58)	35 (61)	24 (42)	0.09
Diabetes mellitus, *n* (%)	15 (26)	7 (12)	9 (16)	0.13
Current smoker, *n* (%)	28 (49)	25 (44)	29 (51)	0.74

DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein; %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerine-induced dilation of brachial artery.
Values are expressed as mean ± SD. Continuous variables were compared by ANOVA and categorical variables by Chi-squared test.

Table 2
Cardiovascular events by tertile of plasma testosterone.

	Tertile 1 <14.2 nmol/L (*n* = 57)	Tertile 2 14.2–19.4 nmol/L (*n* = 57)	Tertile 3 >19.4 nmol/L (*n* = 57)	Total (*n* = 57)
Number of events				
Stroke	2	3	0	5
Coronary artery disease	7	2	2	11
Sudden cardiac death	2	0	0	2
Peripheral arterial disease	1	0	1	2
Total cardiovascular events	12	5	3	20
HRs (95% CI) for total cardiovascular events				
Unadjusted	4.82 (1.36, 17.12)	1.67 (0.40, 6.99)	1(Ref)	
Adjusted for age	6.36 (1.78, 22.80)	1.82 (0.43, 7.71)	1(Ref)	
Adjusted for age and BMI	7.01 (1.94, 25.34)	1.86 (0.44, 7.86)	1(Ref)	

BMI, body mass index. HRs (Hazard ratios) were analyzed using Cox proportional hazards regression.

medications (antihypertensives, statins, hypoglycemic agents and antiplatelet agents), estradiol and DHEA-S. In addition to testosterone, age (HR per year, 1.12; 95% CI, 1.05–1.20), %FMD (HR per 1% increase, 0.80; 95% CI, 0.64–0.99) and HDL cholesterol (HR per 1 mg/dL, 0.88; 95% CI, 0.81–0.95) were independently associated with CV events, but other variables were not in this final model. Further inclusion of other hormones and nitroglycerin-induced endothelium-independent vasodilation into the model did not influence the statistical results (data not shown).

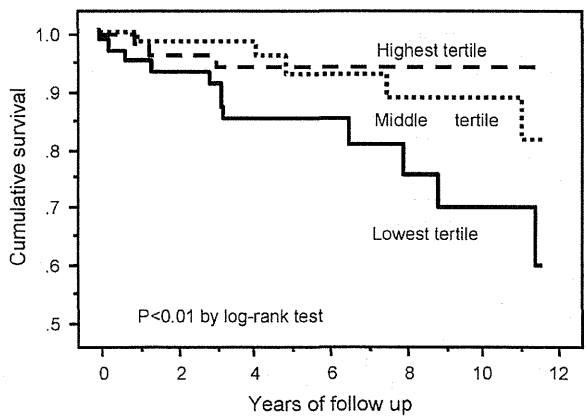

Fig. 1. Survival curves for cardiovascular events by tertile group of plasma concentration of testosterone. Cut-offs of the tertiles for testosterone were 14.2 and 19.4 nmol/L (410 and 560 ng/dL).

Two subjects with the lowest tertile of plasma testosterone suffered CV events within 6 months of follow-up; a case of sudden cardiac death and a case of coronary artery bypass grafting. Accordingly, similar statistical analyses were performed excluding these two cases. The results were essentially unchanged, although the HRs were slightly smaller (unadjusted HR, 3.06; 95% CI, 1.21–7.78: multivariate-adjusted HR, 3.80; 95% CI, 1.06–13.52).

Among other hormones examined, only DHEA-S was associated with increased risk for CV events, but was canceled by adjustment for age (data not shown). Further multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

4. Discussion

In this follow-up study of middle-aged Japanese men with coronary risk factors, a low plasma testosterone level was associated with CV events. Although the subjects with lower testosterone levels had worse profiles of coronary risk factors [4–7,11,12] and endothelial function [8] at baseline, as reported previously, adjustment for these confounders including age and cardiovascu-

lar medication indicated that low testosterone was an independent risk factor for CV events. In contrast, DHEA-S, estradiol and cortisol levels were not related to CV events.

A number of cross-sectional studies have shown an association between low testosterone level and CV disease [17,18], but have not provided evidence of a causal relationship between them. In recent years, longitudinal follow-up studies have demonstrated that community-dwelling older men (around 70 years on average) with lower testosterone levels are more likely to die from CV disease [11,12]. In contrast, a low testosterone level was not associated with CV deaths [19] or events [20] in community-dwelling middle-aged men (early 50s on average). These different findings might arise from the characteristics of the populations such as age and coronary risk factors, duration of follow-up and/or cut-off level of plasma testosterone at baseline. In any case, since all the above-mentioned studies were achieved in Caucasians, our study is the first to investigate the relationship between endogenous testosterone and CV events in Asians. Also, the present study showed a positive association between low testosterone level and CV events in middle-aged men with coronary risk factors, implying the clinical importance of measuring plasma testosterone in patients at risk, even if they are not old.

Unlike the previous reports showing an association of CV events with low levels of DHEA-S [21] and estradiol [22], and with a high cortisol:testosterone ratio [20], the present study did not show any significant association of CV events with estradiol, cortisol or cortisol:testosterone ratio (data not shown). The association between low DHEA-S and CV events was abolished by statistical adjustment for age, suggesting that the age-dependent decline of DHEA-S (Pearson's correlation coefficient between age and DHEA-S: −0.588; $P < 0.001$) might have eliminated the association with CV events if present. Taking together with the Cox regression model including all hormones, it is suggested that testosterone is the strongest among four steroid hormones that could be predictive of CV events in this population.

There could be several mechanisms by which endogenous testosterone protects men from CV disease. Consistent with the present study, observational studies [4–8,11,12] suggest that testosterone might prevent risk factors such as obesity, hypertension, dyslipidemia, diabetes and endothelial dysfunction. Supplementary studies support the beneficial effects of testosterone on adiposity [23] and endothelial vasomotor function [24]. Based on these findings, risk markers and endothelial vasomotor function were entered into the multivariate models. Although statistical adjustment may have been insufficient to exclude the interaction between testosterone and these risk factors, testosterone remained a significant predictor of CV events in the present study. Testosterone has been reported to inhibit vascular smooth muscle cell proliferation and neointima formation [25], suggesting the direct action of testosterone on the vasculature. Also, the effects of testosterone on inflammation, hemostasis and cardiac ischemia [26] might be involved in the final process leading to CV events. The precise mechanisms, including the role of the androgen receptor and aromatization to estrogen, should be addressed in the future.

The finding of this study should not be extended to men without coronary risk factors. Our preliminary data of 47 middle-aged men without coronary risk factors showed that no subject suffered CV events during the mean follow-up period of 102 months, although a quarter of them had plasma testosterone level below the cut-off of this study (<14.2 nmol/L). Thus, the relationship between plasma testosterone and CV outcomes might be totally different in middle-aged Japanese men without coronary risk factors.

This study has several limitations. First, the number of CV events was too small to reach a clear conclusion with strong statistical power, due primarily to the small sample size and secondarily to the low incidence of CV events (approximately 2%/year). Second, the largely retrospective design (the protocol had been approved a few years before the final data collection) reduced the quality of the study and compelled us to lose many plasma samples and 16 subjects in the follow-up. Third, not all the CV events were confirmed by medical recordings. Two cases (a case in the lowest tertile and another in the middle tertile of plasma testosterone level) were determined according to the phone interview to each patient. Although the exclusion of these two cases did not significantly influence the statistical results (data not shown), self-reported outcomes limit the accuracy of this study. Fourth, the potential influence of medication on plasma testosterone level and on CV events cannot be excluded, although statistical adjustment for each class of drugs did not affect the results. For instance, beta-blockers have been reported to decrease plasma testosterone [27], but were taken by only nine subjects and were not related to testosterone level in our population (data not shown). Fifth, active forms of testosterone such as bioavailable and calculated free testosterone were not measured, because a direct assay of bioavailable testosterone or an assay of sex hormone binding globulin, which is necessary for free testosterone calculation, is not available in Japan. However, since previous longitudinal studies [11,12] have shown an association of total testosterone with CV mortality, the fundamental findings might not have differed if active forms of testosterone had been analyzed.

In summary, a low plasma testosterone level was associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This study is the first to show the relationship between endogenous testosterone and CV events in Asian population, and provides evidence supporting the protective role of endogenous testosterone in the development of CV disease in men.

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γ-Secretase Modulators and Presenilin 1 Mutants Act Differently on Presenilin/γ-Secretase Function to Cleave Aβ42 and Aβ43

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SUMMARY

Deciphering the mechanism by which the relative Aβ42(43) to total Aβ ratio is regulated is central to understanding Alzheimer disease (AD) etiology; however, the mechanisms underlying changes in the Aβ42(43) ratio caused by familial mutations and γ-secretase modulators (GSMs) are unclear. Here, we show in vitro and in living cells that presenilin (PS)/γ-secretase cleaves Aβ42 into Aβ38, and Aβ43 into Aβ40 or Aβ38. Approximately 40% of Aβ38 is derived from Aβ43. Aβ42(43) cleavage is involved in the regulation of the Aβ42(43) ratio in living cells. GSMs increase the cleavage of PS/γ-secretase-bound Aβ42 (increase k_{cat}) and slow its dissociation from the enzyme (decrease k_b), whereas PS1 mutants and inverse GSMs show the opposite effects. Therefore, we suggest a concept to describe the Aβ42(43) production process and propose how GSMs act, and we suggest that a loss of PS/γ-secretase function to cleave Aβ42(43) may initiate AD and might represent a therapeutic target.

INTRODUCTION

Alzheimer disease (AD) amyloid-β42 peptide (Aβ42) and Aβ43 are generated from β-amyloid protein precursor (βAPP) and accumulate in senile plaques (Gravina et al., 1995). Because Aβ is secreted as multiple peptide species with different C termini, intramembrane proteolysis of Aβ by presenilin (PS)/γ-secretase (De Strooper et al., 1998, 1999; Sherrington et al., 1995; Struhl and Greenwald, 1999; Wolfe et al., 1999) does not occur at a unique site. However, because even small elevations in the ratio of Aβ42(43) to total Aβ (Aβ42[43] ratio) in secreted Aβ by PS or βAPP mutations trigger familial AD (Kuperstein et al., 2010; Scheuner et al., 1996; Suzuki et al., 1994), the proteolysis is regulated strictly in this aspect. How the variation in Aβ is generated remains unclear.

How βAPP-CTF is cleaved into Aβ42 is controversial because of conflicting findings. First, cleavage at the ε-site generates primarily long fragments, namely Aβ48 and Aβ49, and is followed by stepwise cleavage of every three amino acid residues starting at the C terminus (Qi-Takahara et al., 2005). Two distinct lines have been proposed for Aβ40 and Aβ42 production. Second, cleavage at the γ-site does not correlate directly with cleavage at the ε-site (He et al., 2010).

The ratio of APL1β28, a surrogate marker of Aβ42, to total APL1β is elevated in the cerebrospinal fluid of patients with sporadic AD, including those in the mild cognitive impairment stage and in those with familial AD (Yanagida et al., 2009). Thus, an increase in the Aβ42 ratio in the brain may play a role in the etiology of most AD cases, and the mechanism underlying the regulation of the Aβ42 ratio is a central issue in understanding AD.

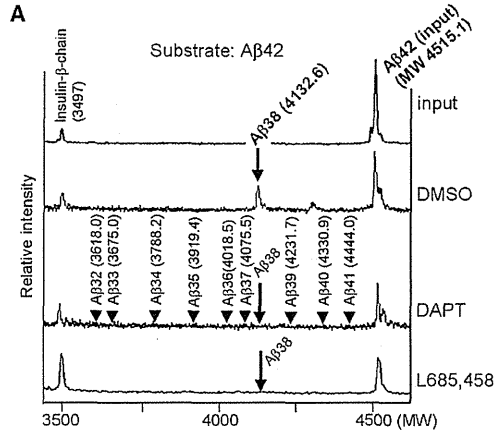
γ-Secretase modulators (GSMs) are disease-modifying drugs that specifically reduce Aβ42 generation (Kounnas et al., 2010; Weggen et al., 2001); some GSMs are being studied in clinical trials. Despite the development of GSMs (Wolfe, 2012), their mechanism of action (Chávez-Gutiérrez et al., 2012) remains unclear.

Given that the VVIA peptide located between the γ38 and γ42 cleavage sites was detected in an in vitro βAPP-CTF cleavage assay (Takami et al., 2009), we asked whether Aβ42(43), formerly considered a product of PS/γ-secretase, could also be a substrate for PS/γ-secretase. Here, we show that Aβ42(43) is an intermediate of PS/γ-secretase, a finding that provides important insight into the regulation of the Aβ42(43) ratio. Our results will help elucidate the mechanism underlying the actions of GSMs and PS mutants.

RESULTS

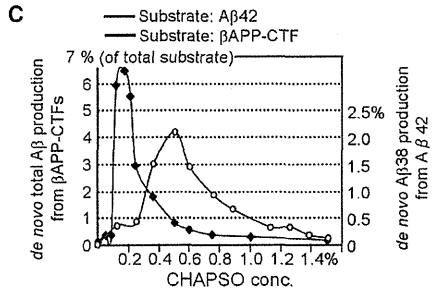
AD-Associated Aβ42 Is a Substrate of PS/γ-Secretase

Initially, we performed a modified in vitro γ-secretase assay in which we used CHAPSO (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate) at its critical micelle concentration of 0.5% instead of 0.25%, the concentration used in the original method (Li et al., 2000). We experimented with using Aβ42 as a substrate instead of the βAPP-C-terminal fragment (βAPP-CTF) (Chávez-Gutiérrez et al., 2012). Surprisingly, Aβ38 was generated de novo from Aβ42 by PS/γ-secretase, a process that we refer to as "Aβ42 cleavage" (Figure 1). MALDI-TOF mass



B Substrate: Aβ42 (1 pmol)

	VVIA	GGVV	GVV	VVI	VIA (fmol)
0 min	1.1	0	0	0	0
10 min	18.1	0	0	0	0.4
20 min	37.4	0	0	0	1.1
30 min	52.5	0	0	0	1.4
L685,458 (30 min)	0	0	0	0	0



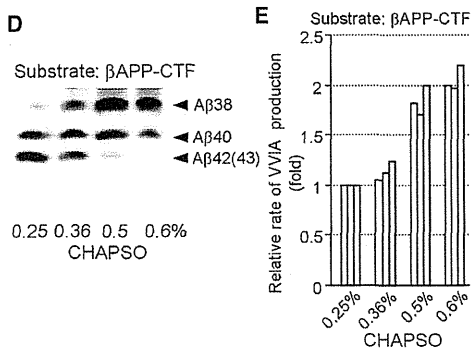


Figure 1. Detection of Aβ and Related Small Peptide Species

Aβ42 and βAPP-CTF cleavage assays were performed at 37°C for 30 min.

(A) Immunoprecipitation-MS analysis of products from the Aβ42 cleavage assay.

(B) Small Aβ-derived peptides (Takami et al., 2009) detected in the Aβ42 cleavage assay.

(C) Levels of total Aβ from βAPP-CTF (black line) and Aβ38 generated from Aβ42 (red line) at various CHAPSO concentrations (0%–1.5%).

(D) Immunoprecipitation-immunoblot detection of Aβ. The volume of each reaction for immunoprecipitation was adjusted to contain the same amount of total Aβ.

(E) Relative rate of the VVIA produced in (D), as defined by [VVIA]/[TVI]. The actual individual data from each of the three experiments are plotted.

See also Figures S1, S2, and S4.

spectroscopy (MS) showed a de novo product of 4132.6 Da, which matches ionized Aβ38 (Figure 1A). Liquid-chromatography tandem MS (LC-MS/MS) (Takami et al., 2009) showed high levels of VVIA tetrapeptide between Aβ38 and Aβ42 (Figure 1B). No further cleavage of Aβ38 was observed, indicating that Aβ38 was the final product (Figure S1A). We also observed a small amount of VIA (~1/40th the amount of VVIA), the tripeptide between Aβ39 and Aβ42, indicating very low-level production of Aβ39 (Figure 1B). Cleavage of Aβ42 was eliminated by γ-secretase inhibitors (Figures 1A and 1B). We also found that the N terminus of the substrate Aβ42 was not necessarily the first residue, because Aβ11–42 was also cleaved (Figure S1B). Collectively, these results indicate that Aβ42 is cleaved into Aβ38 and Aβ39. Irrespective of how cleavage at the γ42-site occurs (Qi-Takahara et al., 2005; He et al., 2010), our current results demonstrate that Aβ42, the pathological PS/γ-secretase product, is also a substrate for PS/γ-secretase.

We next studied Aβ42 cleavage, when βAPP-CTF is the substrate. Cleavage of Aβ42 in the new assay was optimal at ~0.5% CHAPSO, whereas cleavage of βAPP-CTF in the conventional assay was optimal at ~0.2% CHAPSO (Li et al., 2000) (Figure 1C). Given this result, we considered the possibility that the Aβ42 ratio in the de novo Aβ generation in the βAPP-CTF cleavage assay might decrease at a higher CHAPSO concentration (~0.5%). As the CHAPSO concentration increased, the relative ratio of Aβ42(43) production decreased and the ratio of Aβ38 production increased (Figure 1D; Figure S2A). Surprisingly, the relative ratio of Aβ42(43) to Aβ40 was ~0.1 at ~0.5% CHAPSO. Thus, we suspected that the much higher Aβ42(43) ratio produced in the conventional βAPP-CTF cleavage assay than that secreted from living cells may be because the assay was performed in the presence of ~0.25% CHAPSO. In this regard, the in vitro γ-secretase assay in the presence of ~0.5% CHAPSO may be a better model.

Because Aβ42 levels are determined by the balance between its rate of production and its rate of degradation, we measured further the levels of small peptides produced by βAPP-CTF cleavage (Figure 1E). The relative amount of VVIA produced by βAPP-CTF cleavage increased as the Aβ38 ratio increased. These data suggest that the relative levels of Aβ42 are also a function of Aβ42 cleavage. Moreover, CTFγ38/40/42/43, the counterparts of direct cleavage at the Aβ38/40/42/43 sites, have never been observed (Gu et al., 2001). Collectively, our results indicate that Aβ42 is an intermediate stopover product of the cleavage of βAPP-CTF by PS/γ-secretase.

GSMs and Mutant PS1/γ-Secretases Increase and Decrease Aβ42 Cleavage in Living Cells, Respectively

Next, we asked whether the Aβ42 cleavage process plays a role in conditions where the Aβ42 ratio in secreted Aβ changes in vitro and in living cells. First, we examined whether GSMs and inverse GSMs (iGSMs) (Kukar et al., 2005) change the rate of Aβ42 cleavage in vitro when the enzyme activity is low (0.25% CHAPSO) or high (0.5% CHAPSO). Strikingly, in the presence of 0.25% CHAPSO, all GSMs tested (GSM1, Eisai, Compound W, and Sulindac sulfide) increased the relative rate of Aβ42 cleavage (Figure 2A; Table S1A). In contrast, in the presence of 0.5% CHAPSO, the iGSMs tested (fenofibrate and

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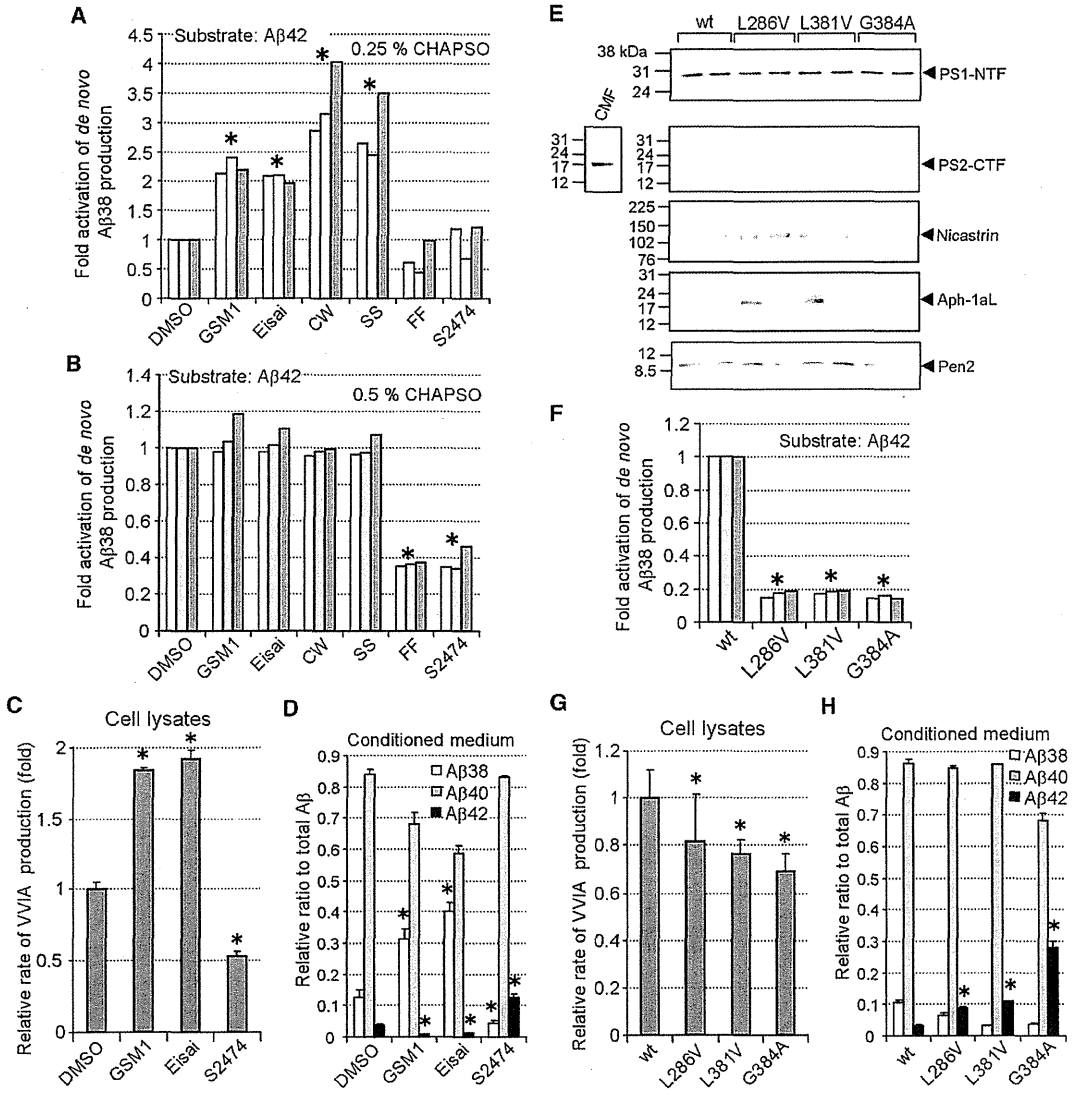

Figure 2. Effects of GSMs/iGSMs and Mutant PS1/γ-Secretases on Aβ42 Cleavage

(A) Fold activation of Aβ42 cleavage by GSMs/iGSMs in the presence of 0.25% CHAPSO. We extracted the PS/γ-secretase fraction from HEK cells stably expressing WT PS1 (Figure S2B). A total of 40 μM GSM1, 10 μM Eisai, 10 μM compound-W (CW), 10 μM SS, 50 μM fenofibrate (FF), or 10 μM S2474 was added to the in vitro assay.

(B) Fold activation of Aβ42 cleavage by GSMs/iGSMs in the presence of 0.5% CHAPSO.

(C) Fold changes of the relative VVIA levels in cell lysates treated with GSMs/iGSM. A total of 4 μM GSM1, 1 μM Eisai, or 30 μM S2474 was added to the cultured medium.

(D) The relative ratio of Aβ species in conditioned medium. The levels of Aβ38, Aβ40, and Aβ42 were measured by ELISA.

(E) Immunoblotting of purified PS/γ-secretase fractions. To show that the same level of each mutant PS1/γ-secretase was used in each reaction, we immunoblotted each fraction with antibodies against all four indispensable elements of PS1/γ-secretase: PS1/2, nicastrin, APh-1-a, and PEN-2. We detected almost equal band densities for all four proteins of mutants and WT PS1/γ-secretase fractions. Exogenous PS1 derivatives displaced the endogenous WT PS2 in the PS/γ-secretase complex. Note that a certain mutant contained a higher level of nicastrin, and thus was omitted from the analysis.

(F) Fold activation of Aβ42 cleavage by purified mutant PS1/γ-secretase.

(G) Fold changes of the relative VVIA levels in cell lysates stably expressing PS1 mutants.

(H) The relative ratio of Aβ species in conditioned medium.

The Aβ38 level was measured by ELISA. Asterisks indicate $p < 0.05$, Welch's t test. Error bars represent SD. The actual individual data from each of the three experiments are plotted in (A), (B), and (F). See also Figures S1 and S2.

S2474) decreased the relative rate of Aβ42 cleavage (Figure 2B; Table S1B).

We do not know how CHAPSO affects the rate of Aβ42 cleavage in vitro. However, in the presence of 0.25% CHAPSO, when Aβ42 cleavage activity is low, iGSMs did not slow the reaction (Figure 2A). Similarly, GSMs did not increase Aβ42 cleavage in the presence of 0.5% CHAPSO, when the activity is high (Figure 2B). These findings suggest that the effects of CHAPSO and GSMs/iGSMs are related.

Next, we investigated whether an increase in Aβ42 cleavage by GSMs is responsible for the decrease in the Aβ42 ratio in living cells. We added GSMs and iGSMs to cultured HEK cells expressing wild-type (WT) and Swedish mutant (sw) βAPP stably, which were the same cell lines used for extracting PS1/γ-secretase for the in vitro experiments. We immediately boiled the treated cells for 2 min to inhibit completely the active degradation of tri-, tetra-, and pentapeptides in the living cells. We extracted the soluble fraction from the resultant cell lysate and measured the levels of the small peptides by LC-MS/MS (Figures 2C and 2G).

Hereafter, the mention of an increase or a decrease in the levels of each peptide associated with the generation of secreted forms of Aβ (e.g., VVIA, VVIAT, and IAT) implies that the relative ratio of a peptide was calculated in relation to that of the sum of peptides associated with Aβ generation (i.e., ITL, VIT, VIV, TVI, IAT, VVIA, and VVIAT) (Figures 2, 3, and 4). GSM-treated cells, which secreted a lower ratio of Aβ42 (Figure 2D; Table S1D), contained a higher ratio of VVIA (produced by cleavage of Aβ42 into Aβ38) than did DMSO-treated cells (Figure 2C; Table S1C). In contrast, iGSM-treated cells, which secreted a higher ratio of Aβ42 (Figure 2D), contained a lower ratio of VVIA (Figure 2C). These data indicate that GSMs and iGSMs increase and decrease, respectively, the rate of Aβ42 cleavage in living cells.

Next, because the Aβ42 ratio in secreted Aβ is increased in most PS1 pathological mutants (Scheuner et al., 1996), we examined whether PS1 mutants integrated into the PS/γ-secretase complex exhibit a reduced rate of Aβ42 cleavage. We extracted the PS/γ-secretase fraction (Figure 2E; see also Figures S2F and S2B–S2D) (Winkler et al., 2009) from HEK cells stably expressing WT or mutant (L286V, L381V, or G384A) PS1 (Figures S2E and S2F). The same amount of enzyme complex was included in each reaction (Figure 2E). We found that the rate of Aβ42 cleavage was lower for mutant than for WT PS1/γ-secretase (Figure 2F; Table S1E), which is consistent with a previous report (Chávez-Gutiérrez et al., 2012). The results are reminiscent of the fact that cells expressing mutant PS1/γ-secretases generally secrete less total Aβ than do WT cells (Shen and Kelleher, 2007). However, it is of note that the reduced rate of Aβ42 cleavage described here is different from the overall loss of function of PS/γ-secretase.

We investigated whether reducing Aβ42 cleavage by mutant PS/γ-secretases could increase the Aβ42 ratio in living cells. We cultured WT or mutant PS1-expressing cells coexpressing sw βAPP (the same cell line used for the in vitro experiments) and analyzed the cell lysates by LC-MS/MS. The relative levels of VVIA were lower in the lysates of mutant cells than in WT cells (Figure 2G; Table S1F). We confirmed the increased Aβ42 ratio in the conditioned medium of the mutant-expressing cells (Figure 2H; Table S1F). These data demonstrate that mutant PS1/γ-secretases decrease the rate of Aβ42 cleavage in living cells. Collectively, our data suggest that the Aβ42 cleavage process is associated with the Aβ42 ratio in secreted Aβ.

A New Concept for the Production of Bona Fide Aβ42

To gain insight into the regulation of Aβ42 cleavage, we next examined how GSMs, iGSMs, and mutant PS1/γ-secretases alter Aβ42 cleavage activity in vitro. The conversion of Aβ42 into Aβ38 can be described by the following scheme and with the following rate constants:

$$[PS/\gamma - secretase] + [A\beta 42] \underset{k_b}{\overset{k_a}{\rightleftarrows}} [PS/\gamma - secretase/A\beta 42 - complex] \xrightarrow{k_{cat}} [A\beta 38] + [PS/\gamma - secretase]$$

This equation can be applied to the production of "free Aβ42" from de novo Aβ42 (shown in the diagram in Figure 3A). Escape from further cleavage and production of free Aβ42 both require that the de novo-generated bound Aβ42 dissociates from PS/γ-secretase. We suggest this concept to explain the production of bona fide Aβ42. According to the model, k_{cat} (unimolecular rate constants) and k_b (dissociation rate constants) values would be relevant to the generation of free Aβ42.

One may think that once βAPP-CTF and PS/γ-secretase form a complex, intermediate long Aβ does not dissociate from the enzyme during the stepwise cleavages. According to the model, the k_{cat} values of the Aβ42 cleavage should vary depending on the substrate. However, without any changes in the relative position of long Aβ species to PS/γ-secretase, the stepwise cleavages might be interrupted. Moreover, we showed clearly that Aβ42, Aβ43, Aβ45, and Aβ46 can bind to PS/γ-secretase and undergo cleavage (Figures 1 and 4). It is unknown whether all of the intracellular Aβ45 and Aβ46 (Qi-Takahara et al., 2005) bind to PS/γ-secretase. Based on these findings, we suggest that the long Aβ species undergoes association/dissociation events with PS/γ-secretase. Therefore, we revised the formulas describing the stepwise cleavage process proposed originally by Takami et al. (2009) (Figure 3B). We introduced the association/dissociation steps clearly for each cleavage step and did not consider that the cleavage at every three amino acid residues is an essential part of the cleavage process. Aβ42 and Aβ43 correspond to $A\beta_{Xn}$ in the revised scheme. Aβ45 and Aβ46 correspond to $A\beta_{X(n-1)}$. Whether the various free $A\beta_{Xn}$ products produced in each step remain at the membrane depends on their physicochemical nature. According to our model, the k_{cat} values of the Aβ42 cleavage should be the same, regardless of the substrate (e.g., βAPP-CTF and Aβ42).

GSMs and Mutant PS1 Increase and Decrease, Respectively, the Velocity at which Bound Aβ42 Is Cleaved to Aβ38 In Vitro

First, to obtain the relative k_{cat} values for Aβ42 cleavage, we performed enzyme kinetic analysis of Aβ42 cleavage over a range of Aβ42 concentrations (Figures 3C–3E; see also Tables S2A–S2C). The Aβ42 cleavage reaction conformed to Michaelis-

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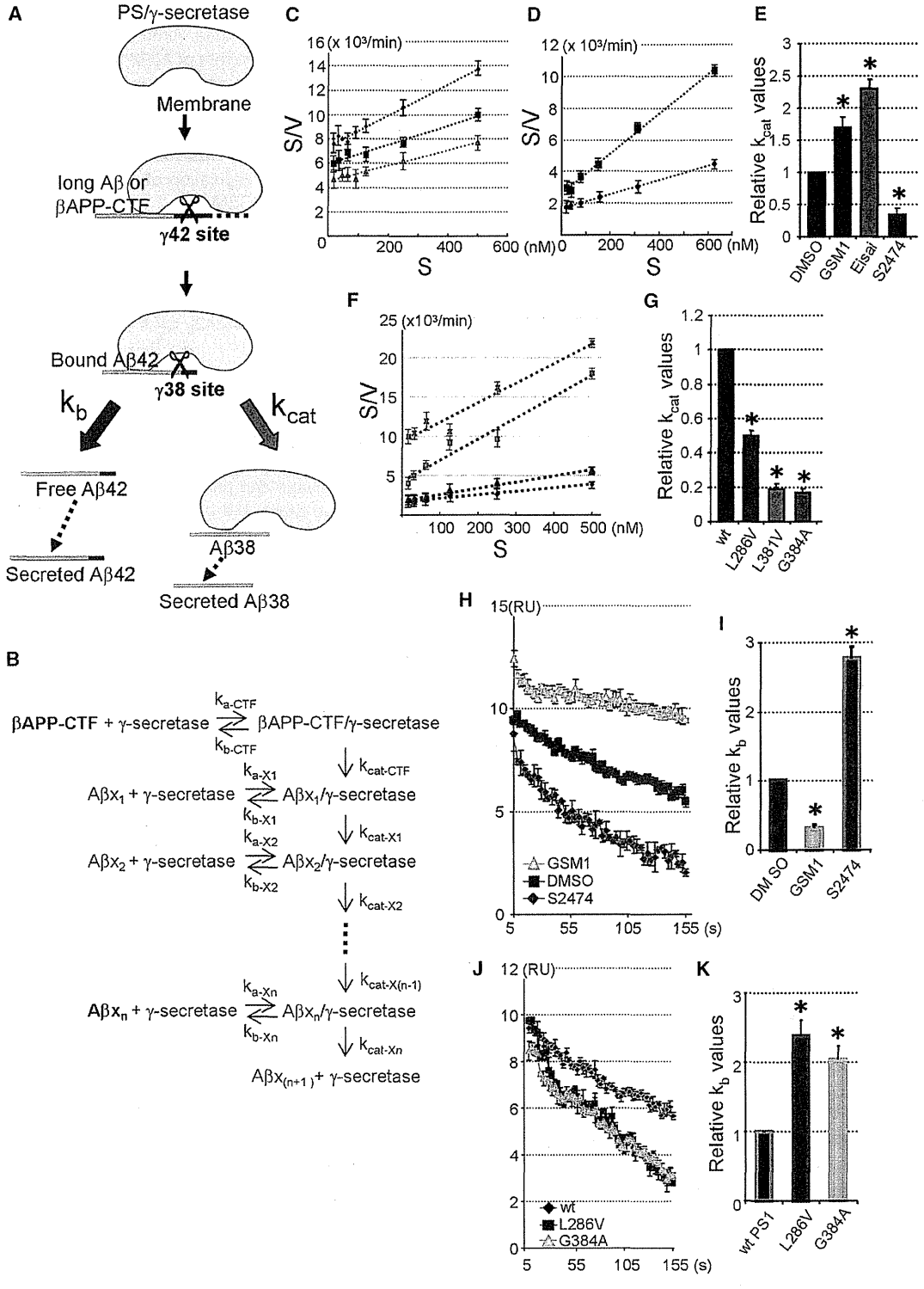

Figure 3. Enzyme Kinetic Analysis of Aβ42 Cleavage and Biacore Analysis of Aβ42 Dissociation from PS1/γ-Secretase

(A) Proposed reaction mechanism for Aβ42 production.

(B) Proposed formulas of stepwise cleavage for Aβ production.

(C) Hanes-Woolf plots of the Aβ42 cleavage in the presence of 0.25% CHAPSO and GSMs (DMSO, black diamonds; GSM1, green squares; Eisai, blue triangles).

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Menten kinetics (Figures S3A–S3D). Next, we examined whether GSM or iGSM alters the k_{cat} for Aβ42 cleavage. Hanes-Woolf plots were used to determine the V_{max} values (Figures 3C, 3D, and 3F). Because Aβ42 was in excess in the reactions, we assumed that $V_{max} = k_{cat}$ [PS/γ-secretase]. The relative k_{cat} value was larger in the presence of GSM1 or Eisai than with WT PS/γ-secretase alone, whereas the value was smaller in the presence of S2474 than with WT PS/γ-secretase (Figure 3E; Table S2C). Depending on CHAPSO concentration (0.25% or 0.5%), the y intercepts of the Hanes-Woolf plots differed, indicating that CHAPSO noncompetitively modified the action of PS/γ-secretase. We next performed reactions using equal amounts of each mutant and WT PS1/γ-secretase (Figure 3F; Table S2D). The relative k_{cat} values were smaller for PS1 L286V, L381V, and G384A/γ-secretase than for WT PS/γ-secretase (Figure 3G; Table S2E). Thus, in the assay conditions used, GSMs increased the velocity at which bound Aβ42 was cleaved to Aβ38, whereas both PS1 mutants and iGSM reduced the velocity of the cleavage.

A GSM Reduces the Velocity at which Bound Aβ42 Dissociates from WT PS1/γ-Secretase, but the Complex of Mutant PS1/γ-Secretases and Aβ42 Dissociates Faster

We also used Biacore binding analysis to measure the relative dissociation rate constant k_b for the complex of Aβ42 bound to PS1/γ-secretase. Aβ42 was immobilized to the sensor tip, and purified PS1/γ-secretases were injected as the analytes. We tried to measure the k_b values to show how fast Aβ42 dissociates from the active center of PS/γ-secretase. We performed the Biacore assay with PS/γ-secretase preincubated in the presence or absence of L685,458, a transition state mimic that blocks the active site of PS/γ-secretase (see Figures S3E–S3J). We assumed that subtracting the resonance unit (RU) value for the L685,458–PS/γ-secretase complex binding to Aβ42 from the RU value for PS/γ-secretase alone (without mixing with L685,458) binding to Aβ42 would give the RU value for PS/γ-secretase, which holds Aβ42 in its active center (see Figures 3H and 3J). Using the RU values, we calculated k_b values for the dissociation of the bound Aβ42 from the active center.

We studied whether GSM1 or S2474 affects the k_b value. During the period from 5 s to 155 s in the dissociation phase, the washout curves of compounds tested with Aβ42 showed simple one-step dissociation with the single exponential rate expected from the model (Figure 3H; Table S2F). The relative k_b values of dissociation in the presence of GSM1 and S2474 were smaller and larger, respectively, than the value for WT PS/γ-secretase alone (Figure 3I; Table S2G). The results indicate that GSM1 and S2474 decreased and increased the rate of dissociation of Aβ42 from the active center of PS/γ-secretase by 0.36 and 2.7 times, respectively, compared with the DMSO control in the assay condition. We also performed similar experiments to measure the relative k_b values for the complex of Aβ42 with WT and mutant PS1/γ-secretase (Figure 3J; Table S2H). The dissociation rates of Aβ42 from L286V and G384A mutant PS1/γ-secretases were 2.4 and 2.0 times larger, respectively, than the rate for WT PS1/γ-secretase (Figure 3K; Table S2I). These data suggest that the velocity at which bound Aβ42 dissociates from PS1/γ-secretase contributes to the changes in the Aβ42 ratio in secreted Aβ caused by the compounds and some mutants. However, we are not yet able to show the extent of the relative effects of the two factors (i.e., k_{cat} and k_b) when the Aβ42 ratio changes in living cells.

Aβ43, Another Long Aβ Species, Is Cleaved into Aβ40 or Aβ38 by PS/γ-Secretase in Living Cells

Aβ43 is another long species of Aβ (Saito et al., 2011). We also investigated whether Aβ43 undergoes further proteolysis in a manner similar to Aβ42. MALDI-TOF MS (Figure 4A) and LC-MS/MS (Figure 4B) showed that Aβ43 was cleaved to Aβ40 or Aβ38 by PS/γ-secretase in vitro. Thus, Aβ38 has multiple precursors. This was confirmed by the in vitro βAPP-CTF cleavage assay (Figure 4C). Why PS/γ-secretase cleaved the substrate at only one of two sites remains unclear. The production of Aβ37 and GVV indicates minor but further cleavage of de novo Aβ40 (Figures 4A and 4B; Figures S1C and S1D).

We measured the amounts of tri-, tetra-, and pentapeptides produced during the stepwise processing of βAPP in living cells (Figure 4D; Figure S4A). Approximately 40% of Aβ38 was derived from Aβ43 (Table S3A). GSMs increased the relative rate of Aβ43 cleavage into Aβ38 (i.e., the level of VVIAT relative to that of total Aβ-related small peptides) (Figure 4E; Table S3B), whereas iGSM and mutant PS1/γ-secretases decreased the rate (Figure 4E and 4F; Table S3C). These results are very similar to the effects of the compounds and the mutants on Aβ42 cleavage (see Figures 2C and 2G; summarized in Figures 4I and 4J). However, both the tested GSM/iGSM (Figure 4G; Table S3D) and some mutant PS1/γ-secretases (i.e., PS1 L381V and G384A) (Figure 4H; Table S3E) decreased the rate of Aβ43 cleavage into Aβ40 (i.e., IAT). Thus, the cleavage of Aβ43 into Aβ38 and that into Aβ40 were affected differently by GSMs.

Next, we asked whether the relative level of Aβ38 derived from Aβ42 and that derived from Aβ43 (i.e., VVIA and VVIAT) in living

(D) Hanes-Woolf plots of Aβ42 cleavage in the presence of 0.5% CHAPSO and iGSM (DMSO, black diamonds; S2474, red squares).
(E) The relative k_{cat} values (n = 4) of Aβ42 cleavage in the presence of GSMs/iGSM.
(F) Hanes-Woolf plots of Aβ42 cleavage by WT and mutant PS1/γ-secretase (WT PS1, black diamonds; PS1 L286V, red triangles; PS1 L381V, blue squares; PS1 G384A, purple crosses) in the presence of 0.5% CHAPSO.
(G) The relative k_{cat} values of Aβ42 cleavage by WT and mutant PS1/γ-secretase.
(H) Fitted curves of dissociation for DMSO (purple/black), GSM1 (18 μM, green/gray), and S2474 (135 μM, blue/red).
(I) k_b values (n = 3 for each) in the presence of GSM/iGSM treatment compared with those obtained in the presence of DMSO treatment.
(J) Fitted curves of dissociation (WT PS1/γ-secretase, blue/black; PS1 L286V/γ-secretase, purple/red; PS1 G384A/γ-secretase, green/gray).
(K) k_b values of mutant PS1/γ-secretases relative to those of WT PS1/γ-secretase.
Asterisks indicate $p < 0.05$, Welch's t test. Error bars represent SD. See also Figure S3.

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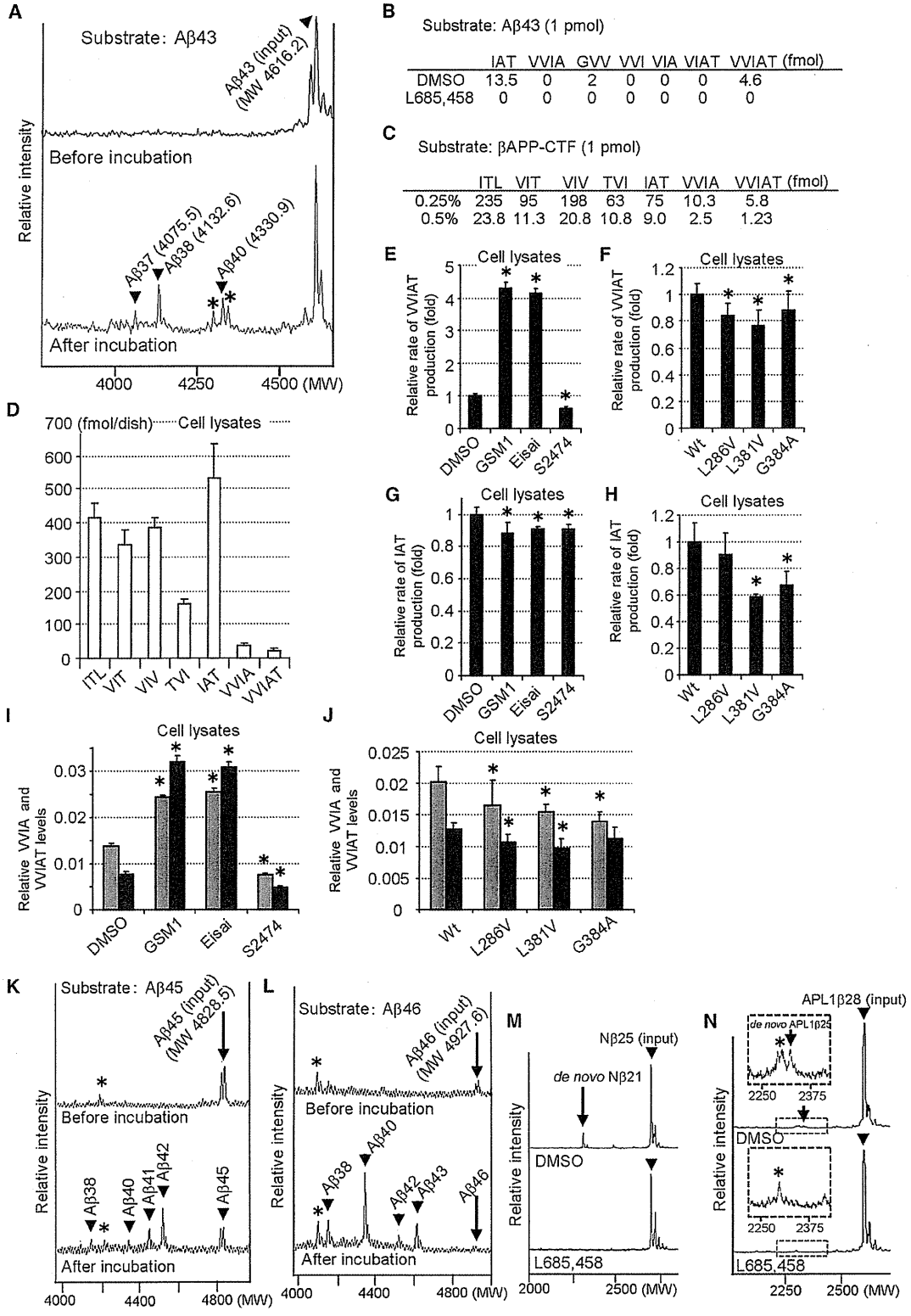

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cells could be changed by the compounds and mutants. GSMs drastically increased the relative levels of both VVIA and VVIAT, whereas an iGSMs decreased the relative levels of the two peptides (Figure 4I; Table S3F). Notably, because of the GSM effect, more than half of the Aβ38 was derived from Aβ43. The PS1 mutants decreased the relative levels of VVIA and VVIAT; however, the degree of the changes was much smaller than that observed for GSM (Figure 4J; Table S3G). The PS1 G384A mutant did not decrease the relative levels of VVIAT. Very similar data were obtained when the levels of VVIA and VVIAT were normalized to the level of each major cleavage in the previous step (i.e., TVI for VVIA and VIV for VVIAT; Figures S4B and S4C). Collectively, these results indicate that GSMs strongly affect Aβ42(43) cleavage, and the increase in Aβ38 production is largely attributed to the cleavage of Aβ43 into Aβ38.

We also performed an Aβ45 and Aβ46 cleavage assay. MALDI-TOF MS showed that both Aβ45 and Aβ46 were cleaved by PS/γ-secretase. Interestingly, Aβ41 and Aβ40, in addition to Aβ42, were produced from Aβ45. Aβ42, in addition to Aβ43, was produced from Aβ46 (Figures 4K and 4L). Combined with the results showing that considerable amounts of Aβ38 were derived from Aβ43, the data indicate that the proposed "Aβ38 product line" (from βAPP-CTF via Aβ48 and Aβ42) and the proposed "Aβ40 product line" (from βAPP-CTF via Aβ49 and Aβ43) (Takami et al., 2009) cross each other.

We studied how the CHAPSO concentration affects the rates of Aβ42(43) cleavage, when βAPP-CTF is cleaved in vitro (Figures S4D and S4E). Interestingly, CHAPSO affected the rates in a dose-dependent manner and in a similar way to the effect of GSMs.

Long Aβ-like Peptides Other Than Aβ Are Also Substrates of PS/γ-Secretase

Aβ-like peptides (Okochi et al., 2002), secreted by a process similar to that for Aβ secretion, include mNotch-1-derived Nβ (Okochi et al., 2006) and APLP1-derived APL1β (Yanagida et al., 2009). We found that PS/γ-secretase cleaved Nβ25 into Nβ21 (Figure 4M) and APL1β28 into APL1β25 (Figure 4N), suggesting that long Aβ-like peptides are generally intermediate products. This may explain why the relative levels of some longer secreted Aβ-like peptides, including Aβ42, change in parallel (Okochi et al., 2006; Yanagida et al., 2009). This finding also indicates that APL1β28 cleavage to APL1β25 is impaired in the sporadic AD brain (Yanagida et al., 2009).

DISCUSSION

In this study, we show that de novo Aβ42(43), a secreted species, is an intermediate of PS/γ-secretase in living cells, and this discovery affects the understanding of the nature of Aβ42(43) production. We suggest that Aβ42 production does not directly reflect the level of cleavage at the C terminus of Aβ42, but rather depends on how much newly produced Aβ42 dissociates from the PS/γ-secretase enzyme and thereby avoids further cleavage. Thus, competition between further cleavage and dissociation from the enzyme may be the key to determining the Aβ42(43) ratio. Importantly, our results also suggest that a new type of partial loss of function in PS/γ-secretase [e.g., reduction in Aβ42(43) cleavage or at the final step of PS/γ-secretase cleavage of βAPP] may cause a gain of function in AD [an increase in the Aβ42(43) ratio]. GSMs increase the relative k_{cat} for the further cleavage of Aβ42 to Aβ38 and decrease the relative k_b for the dissociation of Aβ42 from PS1/γ-secretase. This suggests a potential model to explain how GSMs can lower Aβ42 production.

Chávez-Gutiérrez et al., 2012 showed that PS1 mutations lower the relative levels of Aβ38 to Aβ42 and Aβ40 to Aβ43 compared with WT PS1. The GSMs tested increased both the level of Aβ38 relative to Aβ42 (Weggen et al., 2001) and the level of Aβ40 relative to Aβ43. Based on the hypothetical model proposed by Ihara and colleagues (Takami et al., 2009), those authors speculated that the PS1 mutants and GSMs decrease and increase, respectively, the rate of the fourth cleavage (i.e., Aβ43 cleavage to Aβ40 and Aβ42 cleavage to Aβ38, respectively), possibly because of the premature release of the Aβ42/Aβ43 peptides.

Figure 4. Cleavage of Aβ43, Nβ25, and APL1β28 by PS/γ-Secretase

(A) Representative MALDI-TOF MS spectrum from the Aβ43 cleavage assay (0.5% CHAPSO).

(B) Aβ-derived peptides in the Aβ43 cleavage assay. Addition of L685,458 abolished their generation. Note that Aβ43 cleavage produced much smaller levels of IAT and VVIAT than that of VVIA produced by Aβ42 cleavage. This may be due to the fact that the aggregation property of Aβ43 is higher than that of Aβ42 (Saito et al., 2011).

(C) Aβ species by the βAPP-CTF cleavage assay in the presence of 0.25% or 0.5% CHAPSO.

(D) Aβ species in lysates of HEK293 cells in a 10 cm dish stably expressing sw βAPP and WT PS1.

(E) Fold changes of the relative VVIAT levels in cell lysates treated with GSMs/iGSMs.

(F) Fold changes of the relative VVIAT levels in lysates from cells stably expressing PS1 mutants.

(G) Fold changes of the relative IAT levels in cell lysates treated with GSMs/iGSMs.

(H) Fold changes of the relative IAT levels in lysates from cells stably expressing PS1 mutants.

(I) The relative rates of VVIA (blue) and VVIAT (red) in cells treated with GSMs/iGSMs.

(J) The relative rates of VVIA (blue) and VVIAT (red) in cells stably expressing PS1 mutants.

(K) Representative MALDI-TOF MS spectrum of products from the Aβ45 cleavage assay (0.25% CHAPSO).

(L) Representative MALDI-TOF MS spectrum of products from the Aβ46 cleavage assay (0.75% CHAPSO).

(M) Nβ25 cleavage assay (0.5% CHAPSO).

(N) APL1β28 cleavage assay (0.5% CHAPSO). Insets show an enlargement of the part encircled by the dotted line. Note that APL1β28 cleavage was less efficient than Nβ25 cleavage and Aβ42 cleavage.

Asterisks in (A), (K), (L), and (N) indicate nonspecific peaks, and those in (E), (F), (G), (H), (I), and (J) indicate statistical significance. Error bars represent SD. See also Figures S1 and S4.

We found that, in living cells, ~40% of Aβ38 was derived from Aβ43. Moreover, Aβ40 and Aβ41 were produced in the Aβ45 cleavage assay, and Aβ42 was produced in the Aβ46 cleavage assay. Thus, the putative Aβ38 and Aβ40 product lines (Takami et al., 2009) turn out to overlap at several points.

Takami et al. (2009) showed that Sulindac sulfide decreased the levels of Aβ42 and Aβ43 in a βAPP-CTF cleavage assay, but did not significantly increase the levels of VVIA and IAT. This may be because Sulindac sulfide exerts a weaker GSM action than GSM1 and Eisai, which were used in this study.

We also found that Notch-1 and APLP1 transmembrane domains are cleaved in a similar way, which should help clarify the physiological process of intramembrane proteolysis by PS/γ-secretase. We showed previously that Aβ42 ratio changed in parallel with APL1β28 ratio, and that the APL1β28 ratio increases in the cerebrospinal fluid of AD patients (Yanagida et al., 2009). In this article, we demonstrated that both Aβ42 and APL1β28 were cleaved similarly into the shorter species (i.e., Aβ38 and APL1β25). Therefore, we suggest that Aβ42 cleavage may also decrease in AD brains.

Collectively, we speculate that the increase in the Aβ42 ratio simply reflects the accelerated dissociation of membrane-bound long Aβs (ie, Aβ44~49) from PS/γ-secretase. Because long Aβs on the membrane may perturb the physiological function of neuronal cells, further studies are necessary to investigate whether the prolonged stay of long Aβ at the membrane is pathologically relevant.

At present, inhibition of PS/γ-secretase activity using agents such as Notch-sparing inhibitors is the central approach to decreasing Aβ production specifically. Our results may shift the nature of new drugs for treating AD to repair or increase the ability of PS/γ-secretase to cleave Aβ42(43).

EXPERIMENTAL PROCEDURES

Aβ and Aβ-like Peptide Cleavage Assays

In vitro γ-secretase assays (Li et al., 2000; Osawa et al., 2008) using Aβ and Aβ-like peptides (Aβ42, 43, 45, 46, Nβ25, and APL1β28) were performed under the modified conditions described here with a modified reaction buffer (150 mM citrate buffer [pH 6.0], 0.25 M sucrose, 0.04%–1.5% CHAPSO, 0.1% phosphatidylcholine, 10 μM bestatin, 10 μM amastatin, 5 μM phenanthroline, 10 μM captopril, and 5× Roche protease inhibitor mix).

Extraction of Tri-, Tetra-, and Pentapeptides from Living Cultured Cells

HEK cells stably expressing sw βAPP and PS1 derivatives were cultured to confluence in 10 cm dishes, and 24 hr before collection, the cells were treated with GSM or iGSM. Proteasome inhibitors (1 μM lactacystin, 100 nM MG262, and 1 μM epoxomicin) were added, with or without GSM/iGSM, to the conditioned medium for the last 4 hr. The cells were washed quickly with ice-cold PBS and then immediately boiled for 2 min. The boiled samples were sonicated for 5 s three times and ultracentrifuged. The resultant supernatant was subjected to LC-MS/MS analysis to measure the tri-, tetra-, and pentapeptides.

For additional details, please refer to the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.028.

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ORIGINAL ARTICLE

Classification of delusions in Alzheimer's disease and their neural correlates

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Key words: *Alzheimer's disease, delusions, factor analysis, neuroanatomical basis, regional cerebral blood flow, single-photon emission computed tomography.*

Abstract

Background: Previous findings on neural correlates of delusion in Alzheimer's disease (AD) have been inconsistent because of methodological issues, such as treating multiple delusions as a single entity. In this retrospective study, we classified AD delusions and investigated their neural correlates by using single-photon emission computed tomography data.

Methods: We selected AD patients with delusions from our consecutive outpatients from 2004 to 2010. In this study, eight types of delusions were evaluated with Neuropsychiatric Inventory and classified by factor analysis. Twenty-five of the patients also had single-photon emission computed tomography data, which we used to assess the relationships between cerebral regions of hypoperfusion and hyperperfusion and each classified delusion. The relations were assessed using Statistical Parametric Mapping with normalization to the white matter cerebral blood flow.

Results: The delusions were classified into three factors. Factor 1 consisted of a belief that his/her house is not his/her home, phantom boarder symptom, delusion of abandonment, and belief that one's spouse or others are not who they claim to be. Factor 1 was related to hypoperfusion in the right temporal pole and hyperperfusion in the medial frontal and precentral regions. Factor 2 consisted of delusion relating to the television and delusion of persecution. Factor 2 was related to hypoperfusion in the precuneus and hyperperfusion in the insula and thalamus. Factor 3 consisted of delusion of abandonment and delusional jealousy. Factor 3 was related to hypoperfusion in the right inferior temporal and frontal regions and hyperperfusion in the middle frontal gyrus, insula and posterior cingulate gyrus. Delusion of theft was not included in any factors, and it was related to hypoperfusion in the bilateral thalami and left posterior cingulate gyrus and hyperperfusion in the left inferior frontal regions and anterior cingulate gyrus.

Conclusions: Delusions in AD were classifiable, and each classified delusion was related to different neural networks.

INTRODUCTION

A wide range of neuropsychiatric symptoms and behavioural changes, known as behavioural psychological symptoms of dementia (BPSD), can emerge in the course of Alzheimer's disease (AD).[1,2] BPSD can encompass delusions, hallucinations, agitation, dysphoria, anxiety, euphoria, apathy, disinhibition, irritability and aberrant motor behaviours.[1] Among BPSD, delusions are more likely to appear at the earlier stage and are one of the common symptoms.[3–6] The occurrence of delusions in AD is generally a sign of a worsening prognosis.[7,8] Delusions increase a patient's sense of distress and the burden on the caregivers,[9] and can be a predictor of the need for

institutionalization.[10–12] Current neuroleptic drugs for delusions are not very effective.[10,13] Designing more effective drugs would be easier if the mechanisms by which delusions in AD develop were better understood.

Although many studies have investigated the neural correlates of delusion in AD with single-photon emission computed tomography (SPECT) and positron emission tomography (PET), there is no consensus on these findings.[4,6,14–18] The lack of consensus could be the result of methodological differences, which fall into four categories.

First, previous AD delusion studies considered the delusions as a single entity. However, many types of delusions have been described in AD. The most common types are delusion of persecution,[4–6,11,14,16,17,19–21] delusion of theft,[4–6,11,17,19–22] delusion of abandonment,[4–6,19–21] phantom boarder symptoms (PBS) (belief that some people are in his/her house although the no one is actually there),[4–6,19,20,23] misidentification of people,[6,17,20] including Capgras phenomenon,[14,16,20,21,24,25] belief that his/her house is not his/her home,[4–6,11,17,20,21,24] delusions relating to the television (i.e. the belief that television or magazine images or reports are actually present in the home),[4–6,19,20,23,26] and delusional jealousy.[4–6,14,20,21] Other delusions, such as misidentification of mirror image and the belief that a deceased family member is still alive,[17,19,20,23] can be also observed in AD. Patients with AD often experience more than two types of delusions at the same time.[5] However, AD patients with delusions do not experience all kinds of delusion in the course of the disease, and the frequency of each type of delusion differs.[4–6] Therefore, we thought that delusions in AD may be classifiable and distinguishable neuropsychiatric symptoms.

Second, among the three accumulative radiopharmaceuticals used to assess regional cerebral blood flow (rCBF) with SPECT (technetium-99-labelled hexamethylpropyleneamine oxime, technetium-99-labelled ethyl cysteinate dimer, and iodine-123-labelled N-isopropyl-p-iodoamphetamine (^{123}I-IMP)),[27] ^{123}I-IMP shows the best linearity between the cerebral radioactivity and cerebral blood flow (CBF).[28] Furthermore, ^{123}I-IMP is more sensitive to abnormalities in brain perfusion than the others.[29] However, no studies have yet used ^{123}I-IMP SPECT to investigate the relationship between AD delusions and rCBF. We expected that ^{123}I-IMP SPECT would detect minor alterations of rCBF that previous studies have missed.

Third, most previous neuroimaging studies investigating AD delusions used the regions of interest (ROI) technique to evaluate alterations in rCBF or regional cerebral metabolic rate.[4,14,16,18,24] The ROI technique is not user-independent and cannot evaluate the whole brain. Moreover, the ROI technique does not take individual variations in brain size and shape into account, so it is not suitable for assessing AD brains, which are atrophic. Statistical parametric mapping (SPM) has recently supplanted the ROI technique. SPM analyzes the obtained spatially normalized brain images on a voxel-by-voxel comparison without any priori assumptions and evaluates the whole brain.[22]

Fourth, in statistical analyses, normalization is required to reduce intra-individual variation and to sensitively detect disease-dependent patterns of rCBF and regional cerebral metabolic rate in the SPM.[30,31] Usually, the counts per voxel are normalized to the global mean, which normalizes a global CBF for each subject to 50 mL/100 g/min.[30,31] However, the normalization to the global mean falsely increases CBF and cerebral metabolic rate, which are in fact unchanged.[30] Recently, it has been found that normalization to the white matter produced much less biased patterns of CBF than normalization to the global mean.[30,31]

The aims of this study were to classify delusions in AD with a factor analysis (Study 1) and to investigate the neural correlates of each classified delusion with ^{123}I-IMP SPECT (Study 2). We analyzed the SPECT data with SPM and normalized the counts per voxel to the white matter for statistical analyses.

STUDY 1 AND STUDY 2

These studies were carried out in accordance with the World Medical Association's Declaration of Helsinki (2008) and approved by the Research Ethical Committee of Osaka University (Suita, Japan).

Study 1: classification of delusions in AD

Methods

Participants. Eighty-seven AD patients with delusion were entered into this study. None of them lived in a nursing home. They were consecutive outpatients of the neuropsychological clinic in the Department of Neuropsychiatry of Osaka University Medical Hospital

between December 2004 and December 2010. All patients met the following criteria: (i) met the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria for probable AD;[32] (ii) showed evidence of diffuse cerebral atrophy and possible atrophy in the medial temporal lobes on a cranial magnetic resonance imaging (MRI) or a cranial computed tomography; (iii) had no history of other neurological or psychiatric disorders, serious cerebral vascular disorders, brain tumours, brain injuries, or alcohol abuse; (iv) were at least 60 years old at the first visit; and (v) had a reliable caregiver who could evaluate the BPSD. This study carefully excluded patients who showed indication of dementia with Lewy bodies, such as notable fluctuating cognition with pronounced variations in attention and alertness, recurrent visual hallucinations, or spontaneous motor features of parkinsonism.[33]

The mean age of the patients with delusions was 75.7 ± 6.8 years (range: 63–90 years). The number of women exceeded the number of men (65 vs 22). The mean years of education were 11.5 ± 2.8 (range: 6–18 years). Fifty-five patients (63.2%) were receiving donepezil and nine others (10.3%) were receiving antipsychotics (risperidone: four; tiapride: two; quetiapine: two; sulpiride: one). The mean Mini-Mental State Examination score was 17.4 ± 5.3 (range: 3–26).[34] The Clinical Dementia Rating (CDR) was used to evaluate disease stage.[35] The CDR is a five-point scale with the following grades: 0, no symptoms; 0.5, very mild; 1, mild; 2, moderate; 3, severe.[35] The numbers of patients with CDR grades of 0.5, 1, 2 and 3 were 19, 37, 25 and 6, respectively.

Assessment of delusions. The Neuropsychiatric Inventory (NPI) was employed to evaluate BPSD.[36] The NPI has been frequently used in clinical settings and has been shown to be valid and reliable in both Western countries and Japan.[36,37] The NPI contains 10 subscales of BPSD, including delusion. For those symptoms, the caregiver was asked to rate severity from 0 to 3 and frequency from 0 to 4 for each subscale. The NPI composite scores were calculated by multiplying the severity and frequency scores, so the possible composite scores ranged from 0 to 12 for each subscale. In the delusion subscale, eight different types of delusion, which have been reported to be the most frequent in AD,[20,21] can be evaluated in a manner of present or absent. The eight delusions were: delusion of persecution, delusion of theft, delusional jealousy, PBS, belief that one's spouse or others are not who they claim to be, belief that his/her house is not his/her home, delusion of abandonment, and delusion relating to the television (i.e. the belief that television or magazine images and reports are actually present in the home).[36,37] Although the original NPI defines PBS as a belief that unwelcome guests are living in his/her house,[36,37] we also considered the complaint PBS if a patient complained that family members or acquaintances who had already died or left home were in his/her house. We evaluated the eight delusions within the preceding 30 days by interviewing each patient's main caregiver.

Statistical analysis for clinical data and classification of delusions. The eight types of delusions were analyzed with exploratory factor analysis. Before carrying out a principal component analysis, we assessed the suitability of data and the factorability of the correlation matrix by calculating the Kaiser-Meyer-Olkin measure of sampling adequacy and Bartlett's test of sphericity. The principal component analysis was used to analyze the inter-item relationship and to extract the initial factors, and then a Varimax rotation was performed. The number of factors to be retained was determined by examining the eigenvalues exceeding 1.0 and by examining a scree plot. Items with factor loadings ≥0.30 were entered as a factor. In general, dichotomous responses are not appropriate for a factor analysis. However, if a factor analysis is employed to investigate a general clustering of variables and if the underlying correlations among variables are moderate, a factor analysis for dichotomous variables is allowed.[38] A factor analysis accompanies factor scores, which can be used as variables in subsequent statistical modelling, for each factor. We used the factor scores for Study 2. All statistical analyses of the demographic and neuropsychological data were performed with SPSS v. 17.0 (SPSS Inc. Chicago, IL, USA). An alpha level less than 0.05 was considered to be significant for all statistical analyses.

Results

Frequencies of different types of delusion. The mean composite scores of NPI delusion was 4.4 ± 3.4 (range: 1–12). The most common type of delusion was delusion of theft ($n = 47$, 54.0%), followed by PBS