may be dependent on its HS chain structure (e.g., the degree of N- and O-sulfation, acetylation, and the percentage of iduronic acids in the chain). Second, glypican-1 is exclusively accumulated in DIG domains that contain very little, if any, other HSPGs. To our knowledge, this is the first study showing that glypican-1 is concentrated in DIG fractions. Because Aβ also accumulates in DIG domains, as shown in the present and previous studies, such limited microdomains may provide an environment where glypican-1 interacts closely with Aβ. Although it remains to be determined whether glypican-1 and Aβ form complexes in DIG, the presence of glypican-1 and Aβ in DIG may be linked to the pathological observation that Aβ and glypican-1 co-localize in primitive plaques. Thus, it seems possible that glypican-1 plays a specific role in the accumulation of Aβ and the early phase of plaque formation in AD. Another important finding in the present study was that overexpression of glypican-1 resulted in an enhancement of cell death induced by Aβ42 or thapsigargin. Unlike glypican-3, another glypican family member, of which overexpression induces apoptosis in specific cells (46), our results demonstrated that glypican-1 itself has no influence on cell viability. Cell death observed in Swedish APP and glypican-1 co-expressing cells may be due to increased production of Aβ42 because addition of Aβ42 but not non-fAβ40 or fAβ40 resulted in reduced cell viability when glypican-1 expression was induced. Taken together with findings that Aβ42 but not Aβ40 levels were well correlated with glypican-1 levels in DIG domains, glypican-1 might play a negative role in neuronal cell survival by preferential contribution to Aβ42 accumulation. Furthermore, overexpression of glypican-1 in Swedish APP-SH cells made cells more vulnerable to a specific stress. All stress inducers used in this study were reported to activate caspase-3, thus leading to cell death (47, 48); however, overexpression of glypican-1, together with Swedish APP, resulted in an acceleration of only thapsigargin-induced cell death. Therefore, it appears likely that signals through glypican-1, which occur after Aβ binding, may influence the thapsigargin signaling pathway before capsase-3 activation, but may not activate caspase-3 directly or indirectly. Although the signaling pathway via glypican-1 remains to be further investigated, increasing evidence suggests that DIG domains play important roles in signal transduction by functioning as platforms for signaling receptors (49) and because cross-linking of many GPI-anchored proteins in T or B cells induces clustering of components of signal transduction, it is possible that glypican-1 transduces unique signals as described above. The present in vitro binding analyses indicate that non-fAβ had no or little binding to glypican-1, consistent with previous observations (17, 33). Therefore, Aβ oligomers or fibrillar Aβ appear to be required for binding with glypican-1 via HS chains. We observed that a significant proportion of Aβ exists as SDS-stable dimers in DIG. There is a possibility that Aβ fibril formation is initiated by binding to GM1 ganglioside, which is present in DIG domains and has a binding ability to Aβ (50–52), and the fibril formation is further promoted by binding to glypican-1. Although the structure of HS chains that Aβ recognizes is unidentified, the characteristic nature of HS chains, which are made of repeating disaccharides (typically, repeated 40–100 times), may enable plural Aβ oligomers to bind to a single HS chain. Glypican-1 is considered to have three HS chains; therefore, much more Aβ oligomers could bind to one glypican-1 molecule, facilitating Aβ accumulation in DIG domains more readily. The association of glypican-1 and Aβ may be a factor which promotes not only extracellular deposition but also intracellular accumulation of Aβ. Indeed, it was reported that GPI-anchored proteins are internalized and recycled back to the cell surface 3 to 4 times more slowly than other components of the recycled membrane (53), thereby accumulating inside cells for much longer time. Thus, glypican-1-Aβ complex, which becomes resistant to proteases, may be incorporated

and accumulated intracellularly. It is interesting to note that accumulation of intraneuronal Aβ is observed in the brains of mucopolysaccharidosis (MPS) III, a neurodegenerative disease caused by accumulation of HS chains inside cells due to lack of HS chain-degrading enzymes (54, 55). More recently, it was reported that intracellular Aβ42 induces neuron-specific apoptosis (56). This observation is consistent with our previous hypothesis that intraneuronal Aβ might cause apoptosis without extracellular Aβ deposit (28, 57). Thus, it will be of great interest to examine whether glypican-1 facilitates toxicity of intracellularly accumulated Aβ. The present immunohistochemical observations suggest that glypican-1 is more closely associated with primitive-type plaques than other HSPGs, including syndecan-2 and perlecan. Because syndecan-2 was observed to be deposited only in classic plaques, this HSPG does not appear to have a significant role in Aβ plaque formation. We and others recently demonstrated that intracellular Aβ42-positive neurons are present in AD brains (29, 58). It remains to be clarified whether glypican-1 staining is co-localized with Aβ42 in such intracellular Aβ deposits. In summary, glypican-1 binds to Aβ through HS chains and may be involved in the accumulation of Aβ in DIG domains and/or the formation of plaques at an initial stage. Moreover, glypican-1 may act as a negative factor to neuronal cell survival probably by binding with Aβ. From our results, individuals whose expression levels of glypican-1 are relatively high might have a higher risk of AD. It will be necessary to define more precisely the exact role of glypican-1 in these pathological events. In addition, better understanding of normal and pathological functions of glypican-1 may lead to the development of new therapeutic approaches for AD.

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Fig. 1

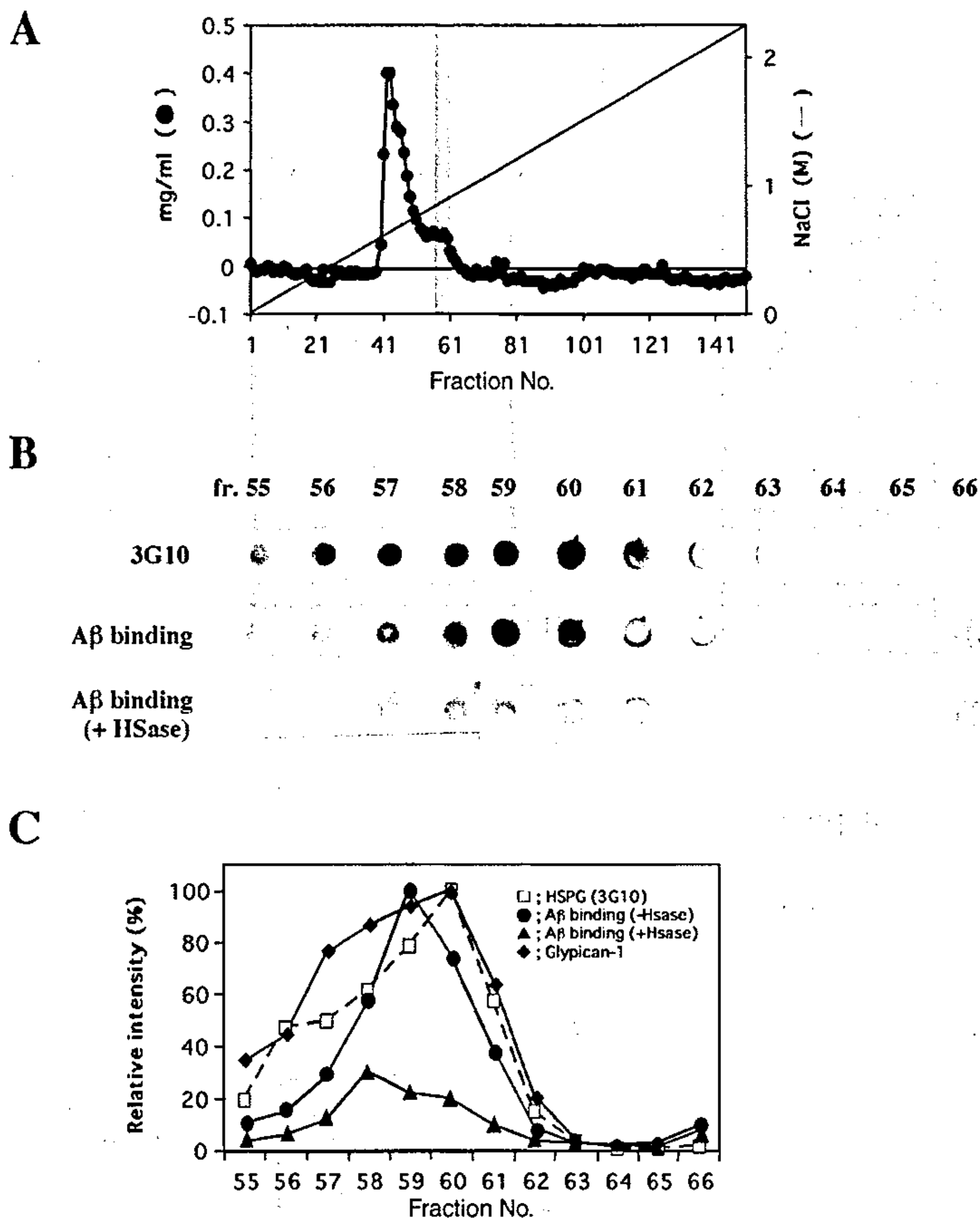


Figure 1. Fractionation of lysates of control human brain, and dot blot Aβ binding assay showing the binding of Aβ to fractions containing HSPGs. (*A*) Control human brain lysates were applied to a DEAE-Sepharose as described in "Materials and Methods". The column was extensively washed with the urea buffer, and then eluted with a linear NaCl gradient (0–2.25 M). One-milliliter fractions were collected, and aliquots were analyzed for protein concentration (solid circles), and assayed for the presence of HSPGs by dot blotting using 3G10 mAb. Shaded area indicates fractions containing HSPGs (fractions 55–61). (*B*) DEAE fractions were spotted onto nitrocellulose membranes. Heparitinase-treated or untreated membranes were incubated with fibrillar Aβ40. Bound Aβ was then detected with BAN50 mAb. (*C*) The intensity of spots was quantitated, and the relative intensity of each spot was plotted. Reactivity of DEAE fractions with 3G10 mAb or anti-human glypican-1 mAb was evaluated using an independent dot blot assay. ●; Aβ binding without heparitinase treatment, ▲; Aβ binding after heparitinase treatment, □; Reactivity with 3G10 mAb, ◆; Glypican-1. All experiments were performed more than two times and reproducible results were obtained. HSase: heparitinase.

Fig. 2

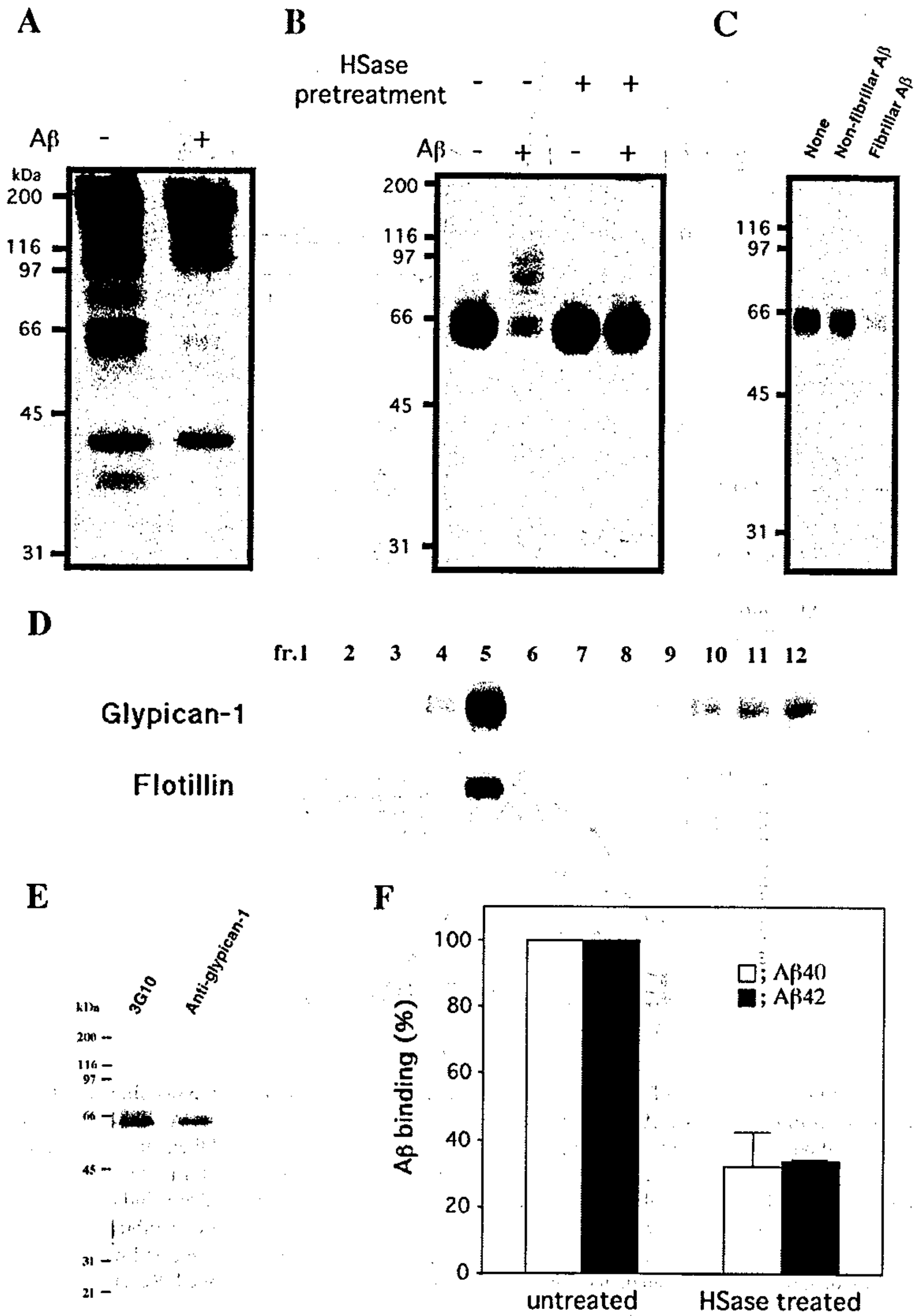
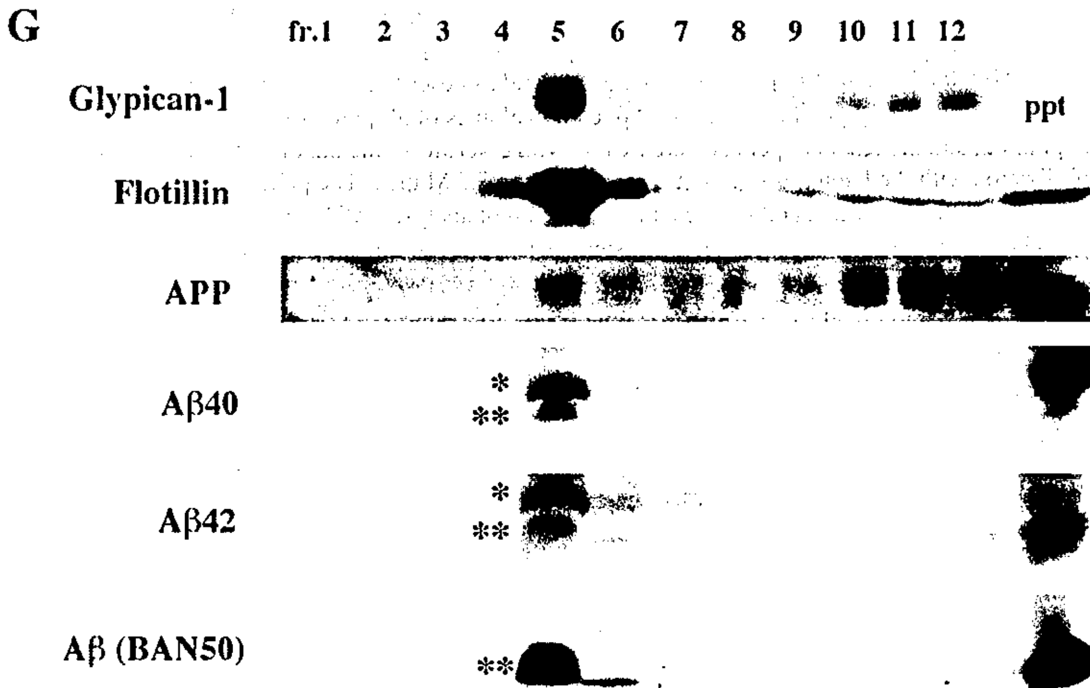


Fig. 2 (cont.)

G



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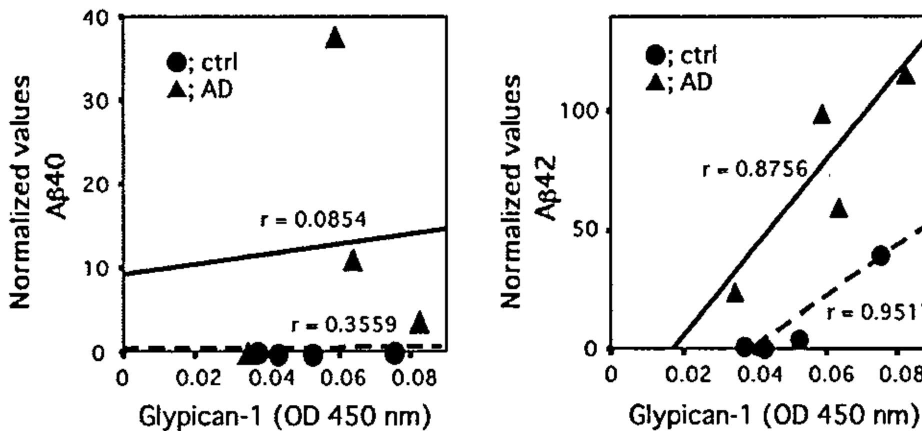


Figure 2. In vitro analysis of Aβ binding to human brain HSPGs in DEAE fractions and the localization of glypican-1 in DIG fractions. (*A*) DEAE fractions containing HSPGs were incubated with or without fibrillar Aβ at 4°C overnight, followed by digestion with heparitinase. Samples were separated by SDS-PAGE, electroblotted, and then probed with 3G10 mAb. (*B*) Heparitinase-treated or untreated control human brain lysates were incubated with or without fibrillar Aβ at 4°C overnight. Samples were digested with heparitinase, and resolved by SDS-PAGE, electroblotted, and then probed with an mAb against human glypican-1. HSase: heparitinase. (*C*) Control brain lysates were incubated with fibrillar or nonfibrillar Aβ40 (1 μg) at 4°C overnight. Samples were treated with heparitinase and analyzed as in panel (*B*). (*D*) Control brain tissue was lysed in 1% Triton X-100 at 4°C and subjected to sucrose density gradient centrifugation as described in "Materials and Methods". Equal volumes of aliquots from each fraction were subjected to Western blotting with specific antibodies to glypican-1 or flotillin. (*E*) The DIG fraction from control human brains was centrifuged, and the pellet was solubilized with 6 M guanidine-HCl. The guanidine-soluble sample was analyzed by

Fig. 2 (cont.)

Western blotting using anti-glypican-1 or 3G10 mAb. Note that almost no HSPG other than glypican-1 was detected with 3G10 mAb. *F*) The guanidine-solubilized samples from the DIG fraction were spotted onto nitrocellulose membranes, and after treatment with or without heparitinase (HSase), Aβ binding was assessed by the dot-blotting assay. Values are expressed as means ± S.E. (*G*) DIG fractions from AD brain were prepared as described in "Materials and Methods". Western blot analysis was performed as in (*A*). In the case of Aβ, equal volumes of aliquots from each fraction were subjected to TCA precipitation, and the resultant pellets were treated with chloroform/methanol, extracted with formic acid, followed by solubilization with the Laemmli sample buffer containing 7.5 M urea. The pellet (ppt) of the sucrose gradients was also solubilized with formic acid and subjected to Western blot analysis. Aβ40 and Aβ42 were detected with FCA3340 and FCA3542, respectively. Single and double asterisks indicate Aβ dimers and monomers, respectively. (***H***) Aβ and glypican-1 levels in DIG domains from control (●) or AD (▲) brains were assessed by ELISA and normalized to levels of flotillin as determined by ELISA. *r*: correlation coefficient.

Fig. 3

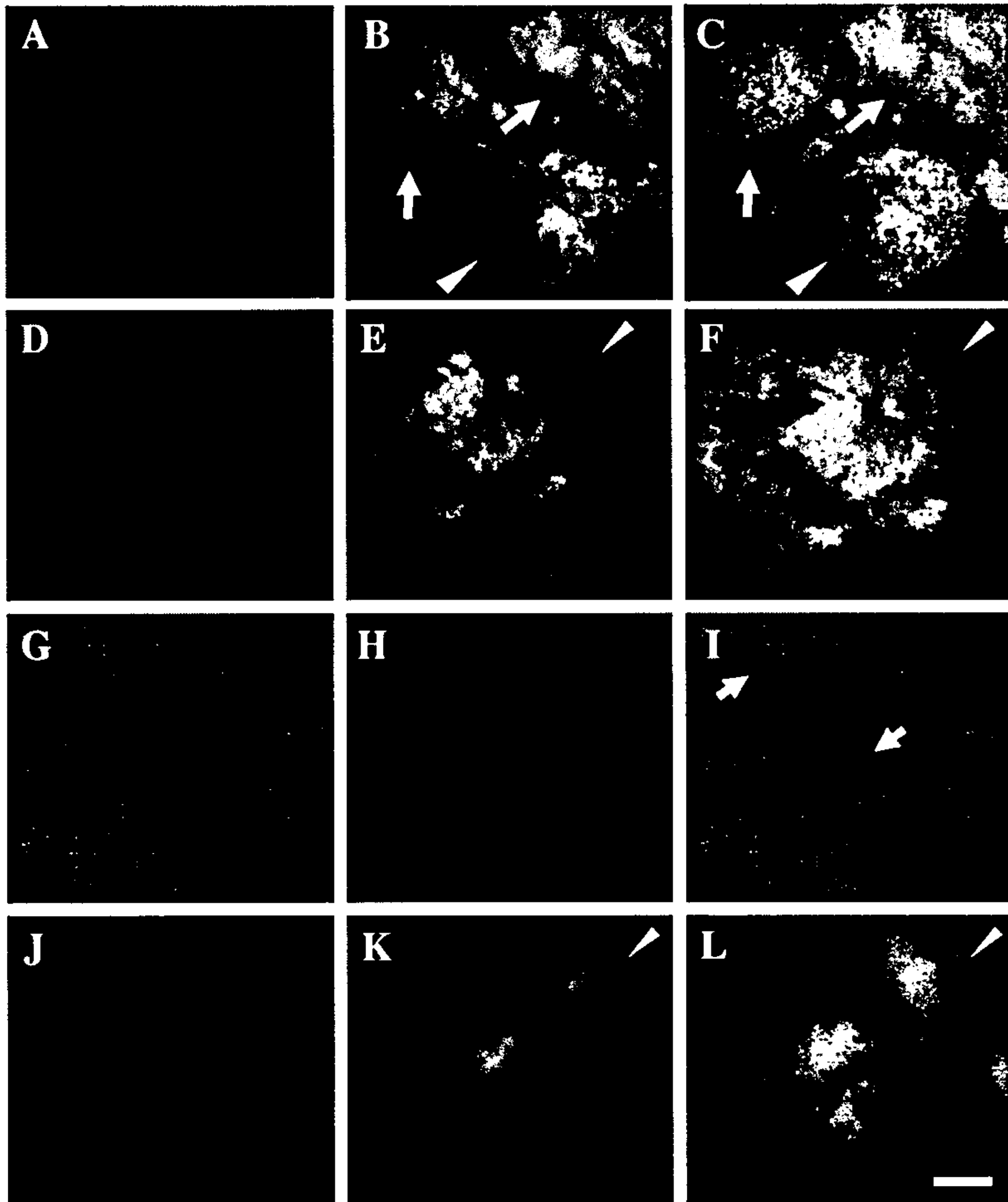
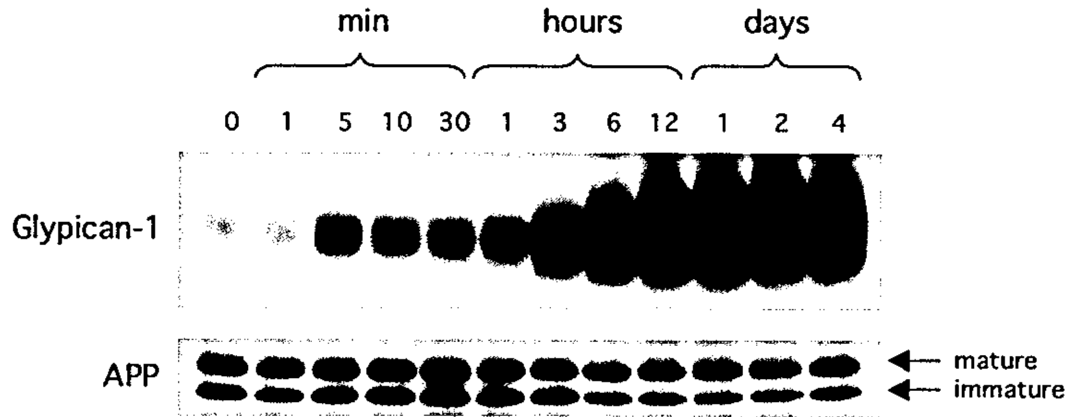


Figure 3. Colocalization of glypican-1 in senile plaques of AD brain. Frozen sections of human AD brain were immunohistochemically stained with antibodies against Aβ42 (*A*, FCA3542), Aβ40 (*D*, *G*, and *J*, FCA3340), glypican-1 (*B* and *E*, S1) and syndecan-2 (*H* and *K*, 10H4), and analyzed by confocal laser scanning microscopy (Fluoview Ver 1.26, Olympus). Texas Red-conjugated (red) anti-mouse IgG and FITC-conjugated (green) goat anti-rabbit IgG were used as secondary antibodies. Colocalization is demonstrated by the yellow signal, generated by the overlay of the red and green signals (*C*, *F*, *I*, and *L*). Primitive plaques, which do not have an amyloid core identified by Congo red staining, are indicated by arrows. Classic plaques with an amyloid core are indicated by arrowheads. Bars, 5 μm.

Fig. 4

A



B

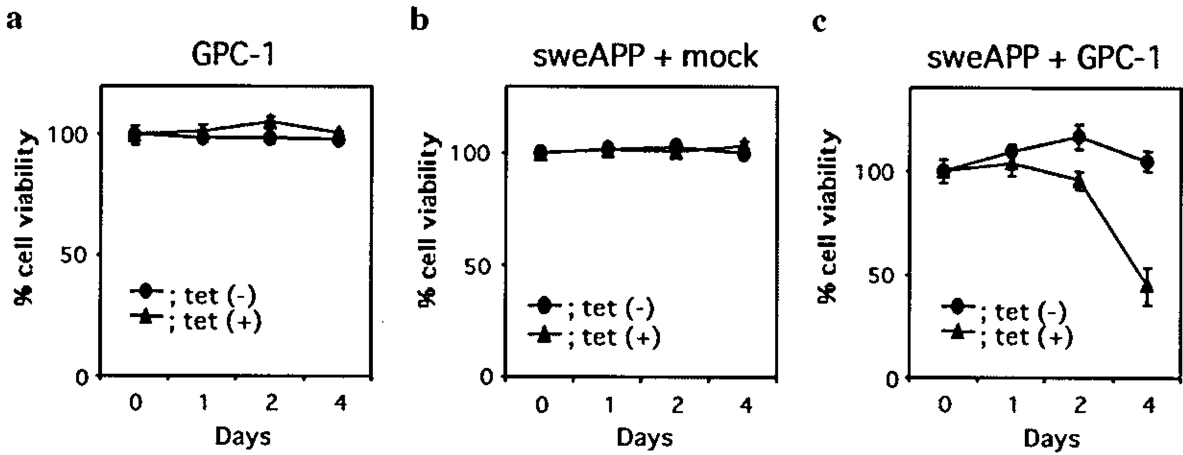


Fig. 4 (cont.)

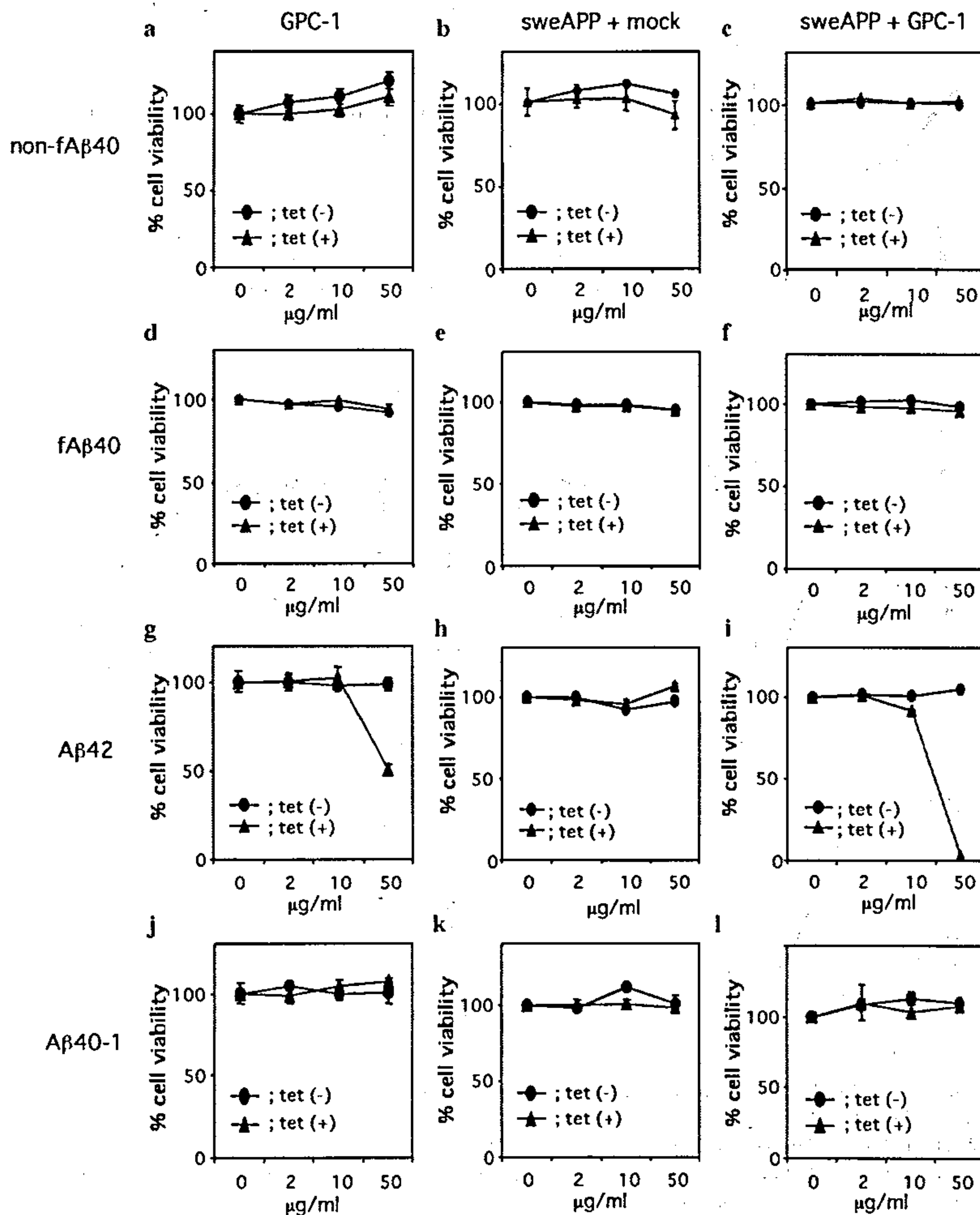


Fig. 4 (cont.)

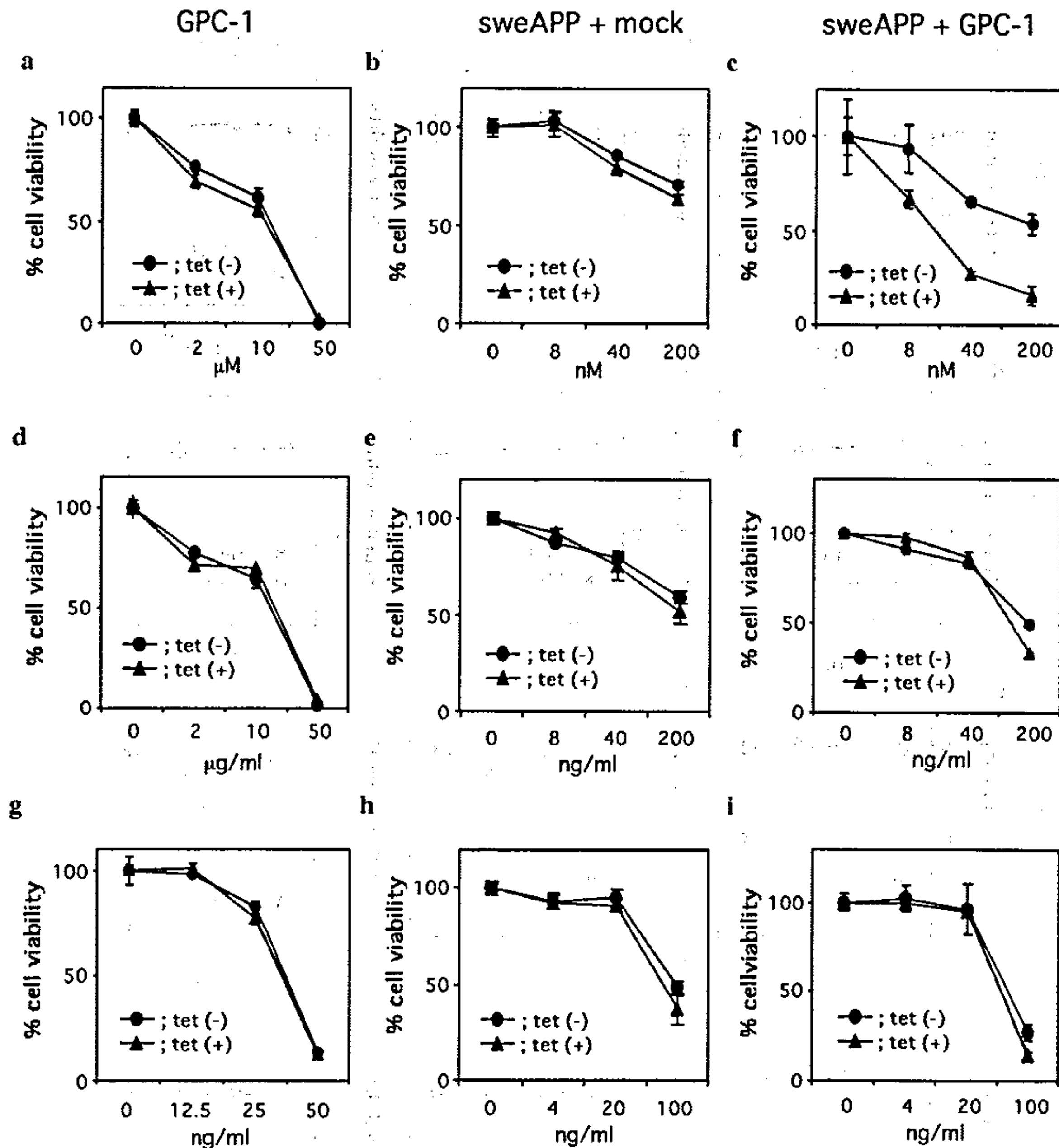


Figure 4. Time course of induction of glypican-1 expression in sweAPP-SH cells and effect of overexpression of glypican-1 on cell viability. (*A*) SweAPP-GPC1-SH cells were cultured with tetracycline for the indicated times and subjected to Western blotting. The same membrane was reprobed by anti-APP Ab as an internal protein control. (*B*) Transfectants were cultured with (▲) or without (●) tetracycline for the indicated times. (*C*) After incubation in medium with (▲) or without (●) tetracycline overnight, transfectants were cultured with non-fAβ40 (*a– c*), fAβ40 (*d–f*), Aβ42 (*g–i*) or Aβ40-1 (*j–l*) in the presence or absence of tetracycline for 4 days. (*D*) After incubation in medium with (▲) or without (●) tetracycline overnight, transfectants were treated with thapsigargin (*a–c*), tunicamycin (*d– f*) or brefeldin A (*g–i*). In (*B–D*), cell viability was monitored by WST assay. Assays were performed in triplicate, and the mean values ± S.E. at 450 nm were measured. Quantitative values are expressed as the percentage of cell viability in untreated cells.

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Molecular basis of Alzheimer's disease: From amyloid hypothesis to treatment in the foreseeable future

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Alzheimer's disease (AD) is the most common dementing disorder in the elderly. It is clinically characterized by insidious onset of memory disturbance followed by slowly progressive global dementia. The patient's brain at autopsy shows diffuse cerebral atrophy, and microscopic findings are characterized by the presence of intraneuronal neurofibrillary tangle (NFT), senile plaques with extraneuronal β amyloid deposits, and dystrophic changes of neuronal processes with synaptic and neuronal loss. The pathological mechanism of these hallmarks are now well understood on the molecular basis, and new strategies to prevent and reverse these pathological changes are now being developed. Here I review some personal interest of the mechanism, and describe future strategies for prevention and treatment of AD.

Keywords: Alzheimer's disease, α-secretase, Aβ vaccine trial, amyloid precursor protein, neurofibrillary tangle, β amyloid.

β amyloid precedes NFT

Neurofibrillary tangle (NFT) is found in neuron soma and dystrophic neurites, which is composed of hyperphosphorylated tau. Activation of phosphorylating enzymes such as GSK-3β, CDK5, and JNK/MAPK by inflammatory stress, abnormalities in the cholesterol metabolism, and aging, or reduction of dephosphorylating enzymes result in hyperphosphorylation of tau, where prolyl isomerase Pin1 is somehow involved.[1] Although it is well correlated with dementia, it is not specific to AD, and dead neurons are much more frequent than NFT-bearing neurons.[2] Moreover, there are several pieces evidence suggesting that βamyloid deposits precede NFT. First, it is well known that senile plaques are the earliest pathological change in AD, which precede 10 years to NFT. Second, injection of βamyloid into the brain of tau-transgenic mice facilitates NFT formation.[3] Thus, the pathological mechanism to cause βamyloid exists upstream of NFT formation.

Amyloid hypothesis

It is now widely accepted that βamyloid has the central role in the pathogenesis of AD (amyloid hypothesis). The reasons are listed in Table 1. The senile plaque is seen in the elderly, otherwise it is specific to AD.[2] The distribution of senile plaques and AD lesions are almost identical.[2] Mutations of familial AD genes such as amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) all resulted in either increased production of total amyloid βprotein (Aβ) or a highly aggregable and toxic form of Aβ composed of 42 amino acid residues (Aβ42).[4] APP transgenic mice show AD-like pathology including all forms of senile plaques, inflammatory changes with microglia activation, synaptic changes, dystrophic neurites, and cognitive dysfunction.[5] A risk factor gene apolipoprotein E (ApoE) enhances senile plaque formation more in the case of E4 than E3.[6] Aggregated Aβ shows neurotoxicity *in vitro and in vivo*, particularly in the senescent condition. Thus, prevention of βamyloid formation or enhanced clearance of βamyloid is a promising way to prevent and treat AD patients (Fig. 1).

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Activation of α-secretase

Aβ is a peptide composed of about 40 amino acid residues, and it is cleaved out from APP by β-secretase and γ-secretase. Since α-secretase cleaves at the mid-portion of Aβ to form a secreted form of APP (Fig. 2), enhancement of α-secretase is expected to slow down the Alzheimer pathological processes. Enzymes that have α-secretase activity in the brain are ADAM-9, ADAM-10, and TNF-α converting enzyme (TACE). It is known that activation of protein kinase C results in activation of α-secretase. Thus, phorbol is the strongest activator of α-secretase, but it not useful for treatment of AD because of its carcinogenic activity. Acetylcholine agonists[4] and estrogen[5] are demonstrated to possess α-secretase activating effects. Recently, sumoylation, a new protein modification is demonstrated to activate α-secretase activity.[6]

Table 1 Amyloid hypothesis in Alzheimer's disease (AD)

1	Senile plaques are seen in the elderly, otherwise specific to AD
2	Distribution of senile plaques and AD lesions are almost the same
3	FAD changes of APP, PS1, PS2 genes are related to increased Aβ production
4	APP transgenic mice represent Alzheimer pathology including senile plaques in the brain
5	ApoE4 increases senile plaque formation
6	β protein is neurotoxic *in vitro* and *in vivo*

Inhibition of β-secretase and γ-secretase

Inhibitors of β-secretase and γ-secretase may be the most promising way to prevent or treat AD patients. Thus, it is now in severe competition among pharmaceutical companies in the world. Several candidates of β-secretase inhibitors or γ-secretase inhibitors have been reported (Fig. 3). However, β-secretase and γ-secretase are not specific to APP. For instance, β-secretase cleaves Golgi-resident sialyltransferase, and γ-secretac is also involved in the cleavage of APP-like protein (APLP), Notch1–4, ErbB-4, E- and N-cadherin, low density lipoprotein receptor-related protein (LRP), CD44, and others. Therefore, inhibitors affect processing of not only APP but also other proteins, resulting in side-effects. Thus, inhibitors specific to APP processing must be sought.

APP-specific inhibitor

Development of APP-specific inhibitors may be possible. Weggen *et al.* reported that ibuprofen and

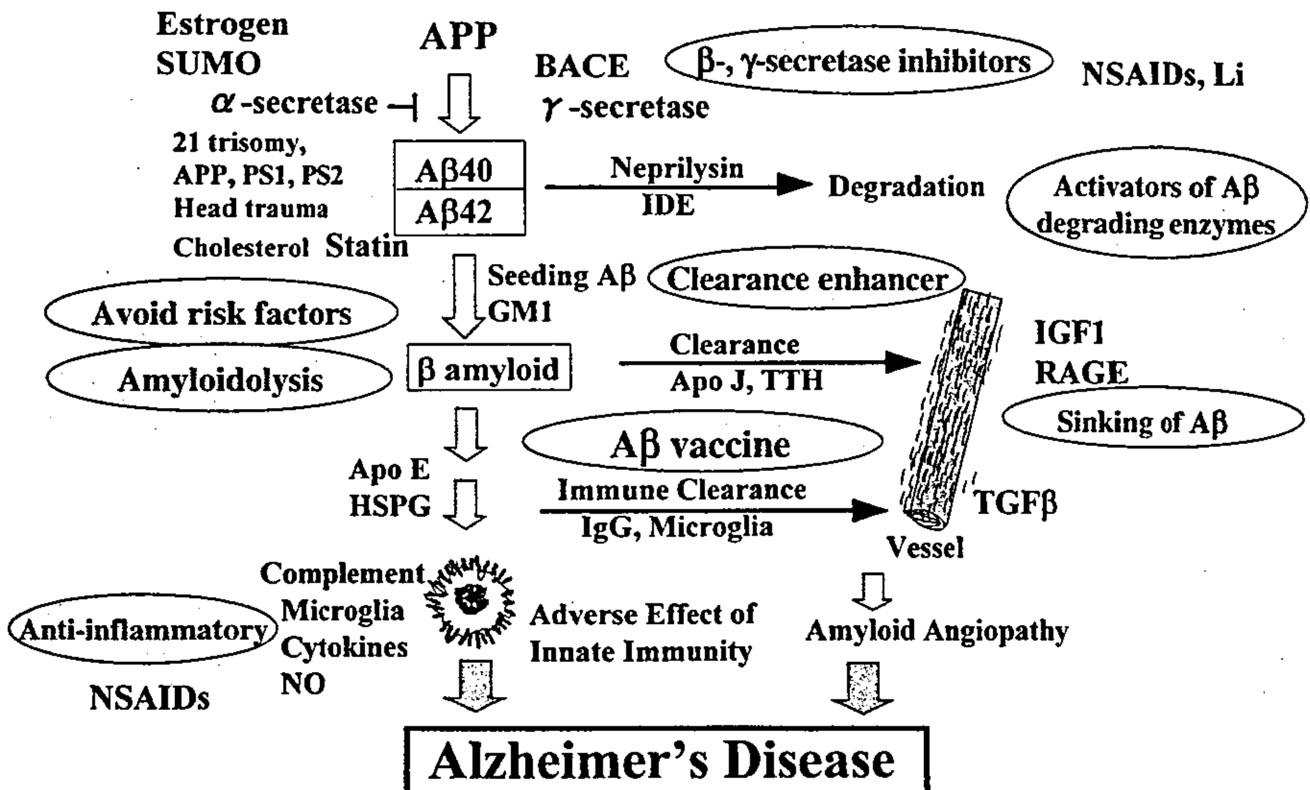


Figure 1 Amyloid hypothesis and strategies for prevention and treatment of Alzheimer's disease. Based on the amyloid cascade, strategies for prevention and treatment of Alzheimer's disease are being developed at each step.

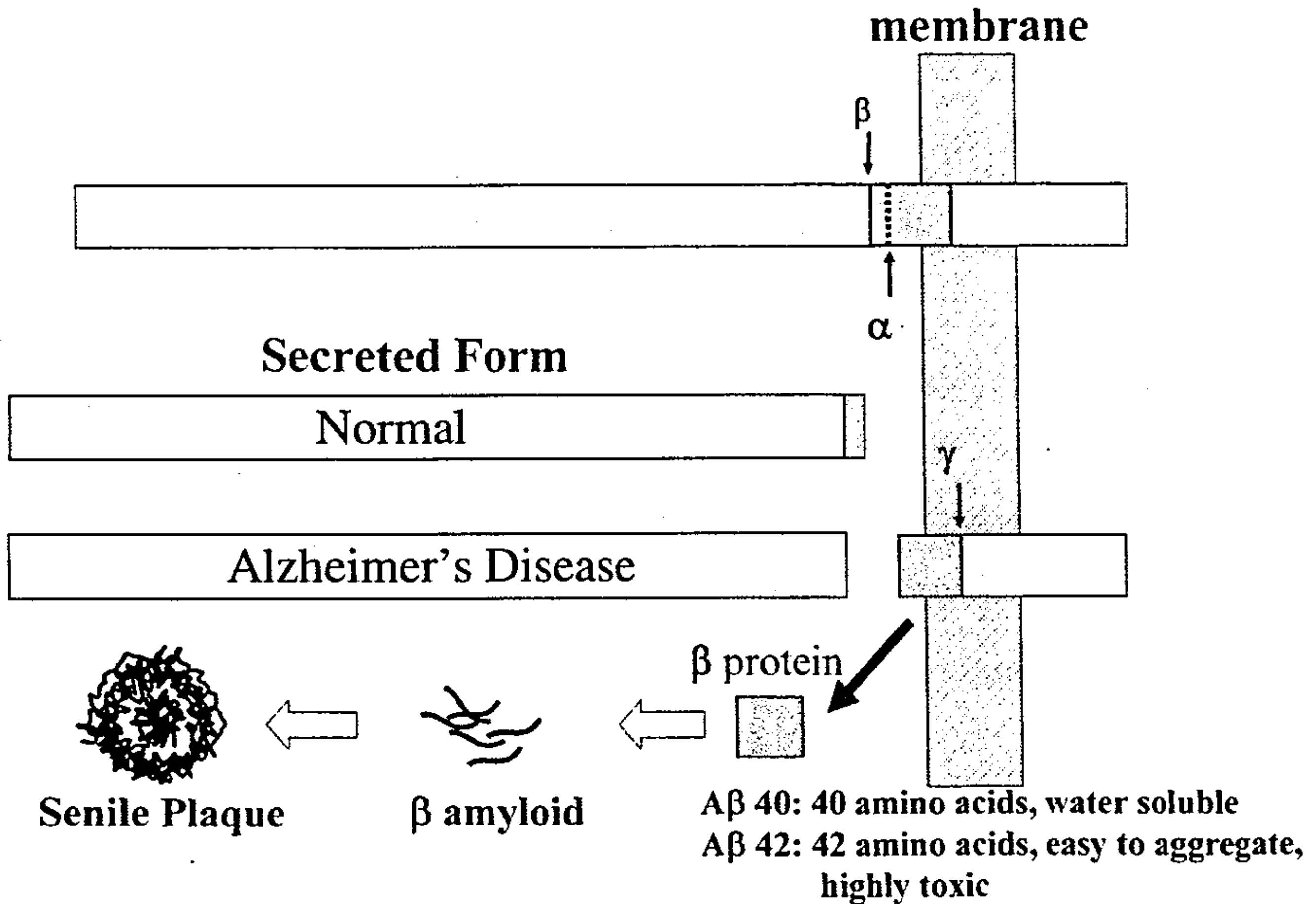
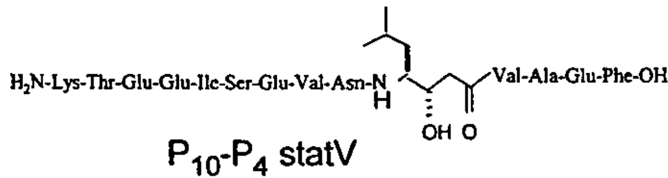


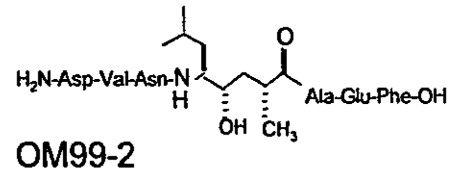
Figure 2 Processing of amyloid precursor protein (APP). APP is a transmembrane protein, which is cleaved by α-secretase to produce a secreted form of APP. In Alzheimer's disease, cleavage at β-secretase and γ-secretase is increased, resulting in overproduction of Aβ, which is in turn aggregated to form β amyloid and deposits in senile plaques

sulindac, a kind of non-steroidal anti-inflammatory drugs (NSAIDs), inhibit Aβ42 production, but not Aβ40, without affecting Notch-1 processing.[7] Phiel *et al.* demonstrated that lithium chloride reduces both Aβ40 and Aβ42 *in vitro* and *in vivo* without affecting Notch-1 processing.[8] In addition, there are several epidemiological studies reporting that NSAIDs users show the significantly lower incidence of AD. Since both drugs are now in clinical use, clinical trials for safety could be skipped in AD, particularly in the case of lithium, which is effective at the dose of clinical use for patients with manic depression. Moreover, if the differential effects of these drugs on APP and Notch are known, the establishment of APP-specific inhibitors seems to be possible. For instance, reduction of Aβ42 by NSAIDs involves Rho,[9] which could be the second target to establish specific treatment. At the very least, secretase inhibitors are the most attractive and promising way of prevention and treatment of AD, but it will take time before these drugs are used in humans.

Immune-mediated clearance of β amyloid

The most foreseeable treatment may be immune-mediated clearance of βamyloid, in other words, vaccination. Dale Schenk and his colleagues first demonstrated that immunization of APP transgenic mice with aggregated Aβ and adjuvant prevented βamyloid deposits, and already deposited βamyloid might be cleared.[10] Similar effects were demonstrated by the passive transfer of Aβ antibodies, and effective antibodies were found to facilitate Fc receptor-mediated phagocytic clearance by microglia.[11] Demattos *et al.* advocated a sink hypothesis.[12] This hypothesis says that Aβ antibody sequestrates peripheral Aβ, causing reduction of periphal Aβ levels, which pulls out Aβ from the brain, resulting in the reduction of Aβ in the brain. The third mechanism is suggested by McLaurin *et al.*[13] They found that epitopes recognized by the effective antibodies exist in Aβ4–10, and these antibodies inhibit Aβ aggregation as well as show lytic activity on amyloid





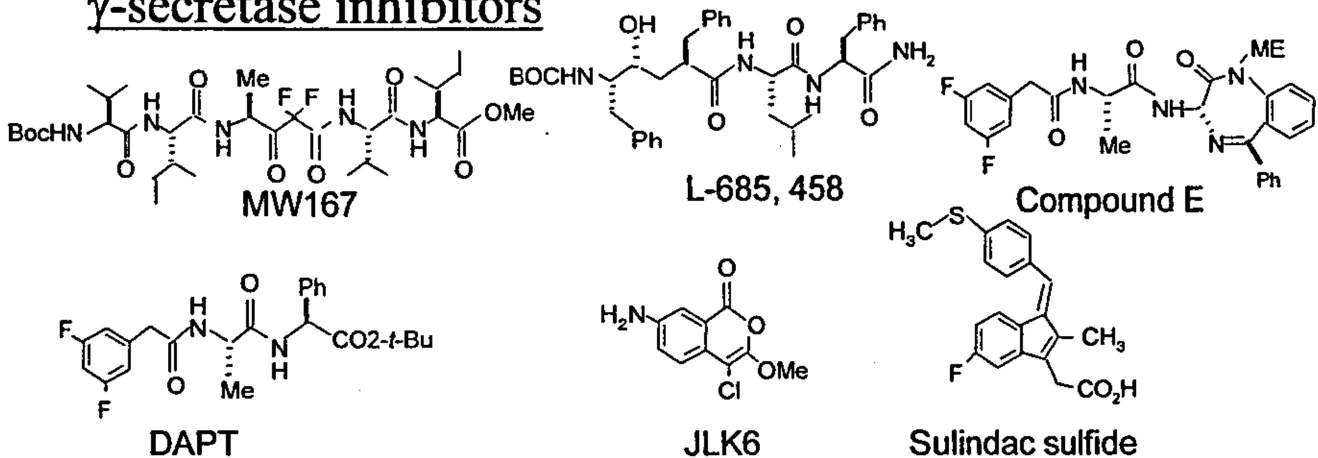


Figure 3 Some chemical structures of β-secretase and γ-secretase. There are several chemicals to show β-secretase or γ-secretase activities.

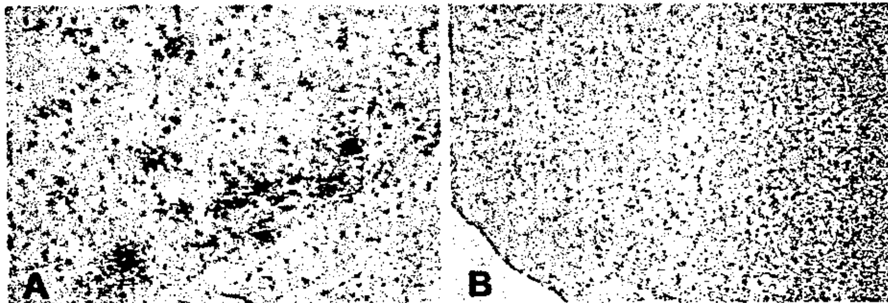


Figure 4 Immunohistochemical staining of mouse brains with an anti-Aβ antibody (4G8).The control brain (A) shows a lot of senile plaques, while the brain of mice which received oral Aβ vaccine shows significant reduction of Aβ deposits (B).

fibrils. Whatever the mechanism is, since treated mice showed improvement of cognitive functions induced by Alzheimer-like pathology of the brain[14] a clinical trial of Aβ vaccine was approved in the USA and Europe.

Aβ vaccine trial

The vaccine AN-1792, composed of aggregated Aβ1–42 and adjuvant QS21, was given to moderately severe AD patients as the phase I trial in 1999 in the US and later in the UK. Since no significant side-effects were noted in the phase I study, a phase IIa trial was started in the US and several countries in Europe in 2000. However, since 15 of the 280 people who received the percutaneous vaccine developed acute meningoencephalitis as a side-effect mostly after the second immunization, the trial was halted.[15] Although the trial was suspended, patients were still followed up. One year after, the patients who developed significant antibodies that recognize senile plaque amyloid showed significantly less decline of cognitive functions than those who did not develop such antibodies.[16] It is also interesting to note that an autopsy case who developed the meningoencephalitis about one year prior to death suggested the disappearance of senile plaque amyloid.[17] In this case, severe amyloid angiopathy and intraneuronal NFTs were observed in the area where senile plaque amyloid deposits were absent. Instead, microglias containing cytoplasmic Aβ were scattered in these regions, suggesting disappearance of senile plaque amyloid by the phagocytosis. It is further interesting to see disappearance of corona-like dystrophic neurites surrounding

classical senile plaques, suggesting the possible remodeling of NFT-bearing dystrophic neurites. Although these clinical and pathological reports are from a single source each, these observations may suggest the efficacy of Aβ vaccination in humans, and development of safer vaccine has been a big subject in the world.

Safe oral vaccine

We developed a safe oral Aβ vaccine using the adeno-associated virus vector (AAV) carrying Aβ1–43 or Aβ1–21 cDNA.[18] When this vaccine was given once into the stomach of tg2576 mice (transgenic mice carrying Swedish-type mutant APP) using an orogastric tube, antibodies to Aβ were elevated efficiently and lasted for over 6 months. The antisera obtained from vaccinated mice stained senile plaque amyloid, and the antibody subtypes were mainly IgG1 and IgG2b. The vaccine was given to mice at 15, 30, and 45 weeks old, and the brain was examined at 56 weeks old. Aβ deposits in the brain were significantly reduced in all vaccinated mice (Fig. 4), and inflammatory cell infiltration was not observed in the brain as well as other systemic organs.

Splenocytes obtained from the vaccinated mice did not show proliferative response to Aβ at all. In the brain of vaccinated mice, activated microglia were increased and GFAP-positive astrocytes were decreased.

We eat meat, eggs, and fish, or drink milk daily. These foreign proteins are digested into small peptides before absorbed, but relatively large peptides or even whole proteins enter into our body by phagocytosis through intestinal epithelial cells. There are numerous antigens in the food that mimic self antigens such as encephalitogenic myelin antigens. For instance, butyrophilin in cow milk mimics myelin-oligodendrocyte glycoprotein (MOG) and immunization with butyrophilin induces autommue encephalomyelitis in animals.[19] The autoimmue encephalomyelitis is mediated mainly by T helper type 1 (Th1) T cells that respond to self antigen. Thus, there exists an immune regulatory system in the gut, where Th1 responses are suppressed and Th2 responses are enhanced.[20] Indeed, our oral vaccine elicited Th2 type immune responses, and resulted in the successful prevention and reduction of βamyloid deposits in tg2576 mice without showing Th1 responses. Our oral Aβ vaccine seems to be beneficial for prevention and treatment of Alzheimer's disease. We are now testing this vaccine in monkeys. When the efficacy and safety of the vaccine are confirmed, a clinical trial will be commenced in very near future.

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