that mutations of Leu166 of PS1 affect the generation of AICD and NICD generation in a similar manner (Moehlmann *et al.*, 2002) suggest that PS is indeed the proteolytic subunit within the multicomponent γ-secretase complex. Such a complex requires additional factors for its assembly, stability and activity. One of these components is nicastrin (Yu et al., 2000), which is required for PS expression (Edbauer *et al.*, 2002; Hu *et al.*, 2002; Lopez-Schier and St Johnston, 2002), γ-secretase activity (Edbauer *et al.*, 2002) and Notch S3 cleavage (Chung and Struhl, 2001; Hu *et al.*, 2002; Lopez-Schier and St Johnston, 2002).

Materials and methods

Antibodies and reagents

The monoclonal antibodies 9E10 against the c-myc epitope and M2 against the FLAG epitope were obtained from Sigma (St Louis, MO). The γ-secretase inhibitor L-685,458, ([(2R,4R,5S)-2-benzyl-5-(Boc-amino)-4-hydroxy-6-phenyl-hexanoyl]-Leu-Phe-NH_2) was purchased from Bachem.

cDNA constructs

The cDNAs encoding the mouse Notch-1 variants NΔE and NICD carrying a C-terminal hexametric myc tag (Schroeter *et al.*, 1998) in pcDNA3-hygro (+) vector were described previously (Steiner *et al.*, 1999a). These Notch-1 variants contain the M1727V mutation (Kopan *et al.*, 1996). Mouse Notch-1 F-NEXT variants either containing or lacking the M1727V mutation were obtained by PCR-mediated mutagenesis. First, F-NEXT M1727V was generated using the ExSite PCR-based site-directed mutagenesis kit (Stratagene) using NΔE as the template and the primers 5′-P-ATCGTCGTCCTTGTAGTCTCTCAAG-CCTCTTGCGCCGAGCGCGGGCAGCAGCGTTAG-3′ and 5′-P-GAC-AAGATGGTGATGAAGAGTGAGCCGGTGGAGCCTCCGCTGCCC-TCGCAGCTG-3′. Subsequently, F-NEXT cDNA was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) using F-NEXT M1727V cDNA as the template and the primers 5′-CCTCGCAGCTGCACCTCATGTACGTGGCAGCG-3′ and 5′-CGCGCCACGTACATGAGGTGCAGCTGCGAGG-3′. Each mutant was sequenced to verify successful mutagenesis.

Cell culture, cell lines and cDNA transfection

K293 cells stably expressing either wild-type PS1 (Okochi *et al.*, 2000), PS1 C92S (Okochi *et al.*, 2000), PS1 L166P (Moehlmann *et al.*, 2002), PS1 L286V (Kulic *et al.*, 2000) or PS1 D385N (Steiner *et al.*, 1999b) were generated and cultured as described. Stable transfections with NΔE, NICD and F-NEXT cDNA constructs were carried out using Lipofectamine 2000 (Invitrogen) according to the supplier's instructions.

Analysis of Notch-1 metabolites

Confluent cells in a 10 cm dish were analyzed for Notch-1 metabolites in pulse–chase experiments. Following starvation in methionine- and serum-free minimal essential medium (MEM) for 40 min, cells subsequently were metabolically labeled with 400 μCi of [^{35}S]methionine/cysteine (Redivue Promix, Amersham) for 1 h in methionine- and serum-free MEM and chased for 2 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and excess amounts of unlabeled methionine. Conditioned media were collected, immediately put on ice and, after a clarifying spin at 3000 *g* and addition of a protease inhibitor cocktail (1:1000; Sigma) and 0.025% of sodium azide, subjected to immunoprecipitation with anti-FLAG M2 agarose (Sigma). Immunoprecipitates were separated on 10–20% Tris-tricine gels (Invitrogen) and analyzed for F-Nβ species by fluorography. Cell lysates were prepared as described (Okochi *et al.*, 2000) and analyzed for NΔE, F-NEXT and derivatives thereof, and NICD by immunoprecipitation with antibody 9E10 as described (Steiner *et al.*, 1999a). γ-Secretase dependence of Notch-1 endoproteolysis was analyzed using γ-secretase inhibitor L-685,458 (1 μM; Shearman *et al.*, 2000), which was added to the culture media 2 h before starvation and present throughout the starvation, pulse and chase periods.

Combined immunoprecipitation/MALDI-TOF MS (IP/MS) analysis of Nβ species

Cell lines stably expressing F-NEXT derivatives were grown in 20 cm dishes. After reaching confluence, the culture media were replaced with 24 ml of 10% FCS/DMEM and media were incubated for 3 h. A 20 ml aliquot of the conditioned media was collected, immediately put on ice and subjected to a clarifying spin. Following addition of a protease inhibitor mix (1:1000, Sigma) and 0.025% sodium azide, conditioned media were immunoprecipitated with M2 agarose for 4 h at 4°C. Immunoprecipitates were washed three times for 10 min at 4°C with wash buffer 1 (0.1% *N*-octylglucoside, 140 mM NaCl, 10 mM Tris pH 8.0, 0.025% sodium azide) and once with wash buffer 2 (10 mM Tris pH 8.0, 0.025% sodium azide). Immunoprecipitated peptides were eluted with trifluoroacetic acid/acetonitrile/water (1:20:20) saturated with α-cyano-4-hydroxy cinnamic acid. The dissolved samples were dried on a stainless plate and subjected to MALDI-TOF MS analysis. The MS peak heights and molecular masses were calibrated with angiotensin (Sigma) and bovine insulin β-chain (Sigma).

Semi-quantitative analysis of F-Nβ species

Conditioned media from the respective K293 cells co-expressing F-NEXT/PS were collected and aliquots of the conditioned media were subjected to IP/MS analysis. The peak heights of F-Nβ1731 in the MS spectra were measured and its peak heights relative to the peak height of 1 pmol bovine insulin β-chain (internal control) were calculated. These relative peak heights were used to calculate the relative levels of the F-Nβ1731 species contained in each conditioned medium of the respective F-NEXT/PS-transfected cells. Subsequently, the amounts of the conditioned media were adjusted to contain the same levels of F-Nβ1731 using a standard curve for F-Nβ1731, and again subjected to IP/MS analysis. After confirming that the F-Nβ1731 peak has the same height as the peak of the internal control, peak heights corresponding to C-terminally longer F-Nβ species (F-Nβ1733, F-Nβ1734 or F-Nβ1735) were measured and its peak heights relative to the internal control were calculated. Relative peak heights of F-Nβ species obtained from endogenous/wild-type PS1 and PS1 FAD-associated mutants were compared.

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

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

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

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

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

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

Intramembranous Endoproteolysis Is Essential for the Novel Signaling Paradigm

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

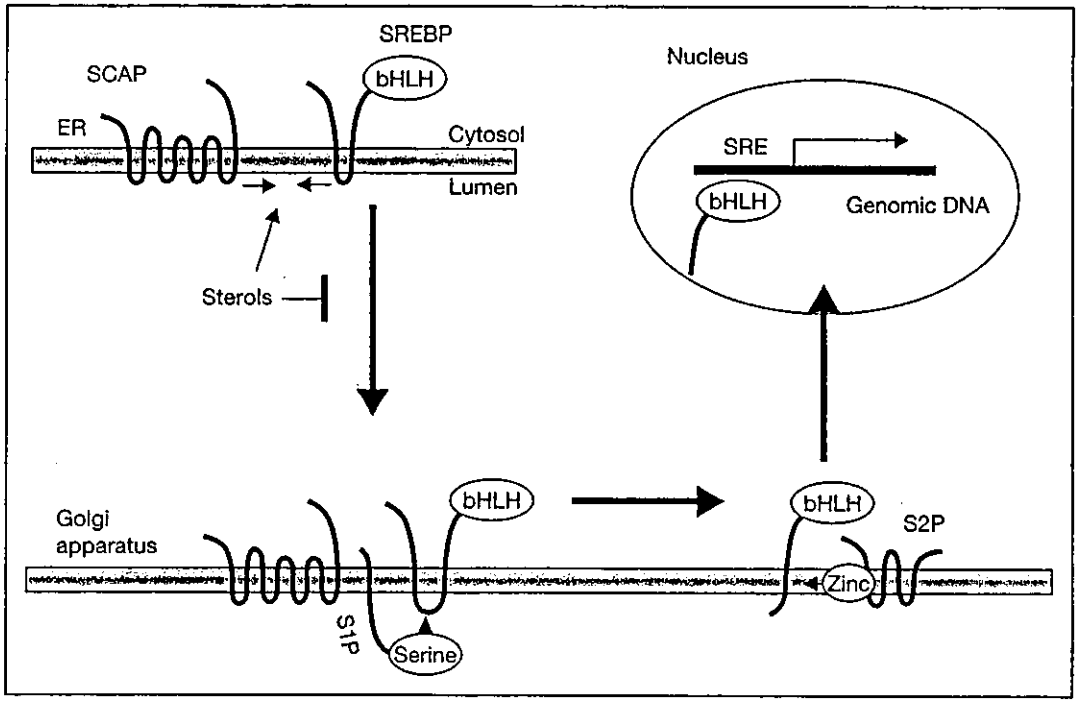

Fig. 1. Endoproteolysis of SREBPs: Concept of RIP. Serine of S1P indicates a proteolytic active center and zinc of S2P metal ion binding to the active center.

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

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

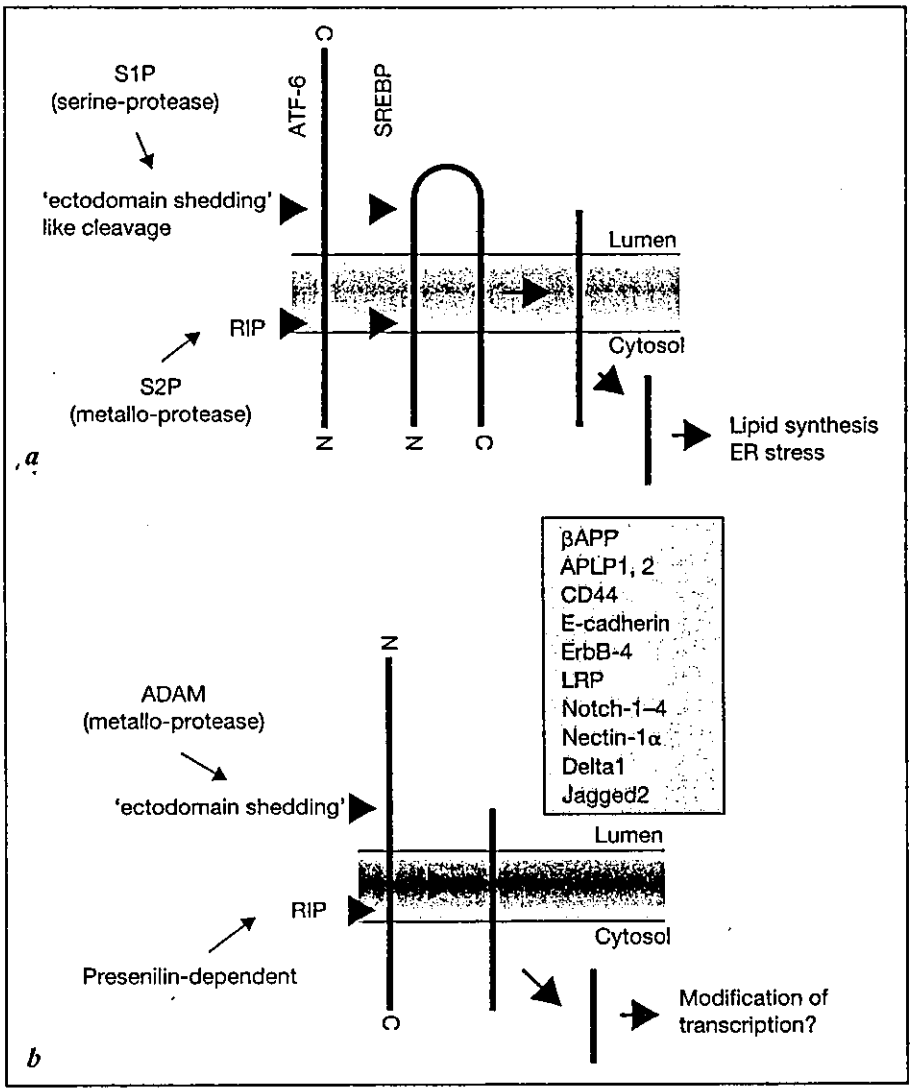

Fig. 2. ***a*** Sequential cleavages of ATF-6 and SREBPs share common features. ***b*** Common sequential cleavage mechanism (RIP) when substrates share the type-1 topology in their transmembrane domain.

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

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

Table 1. List of putative polytopic TM I-Clips

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

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

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

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

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

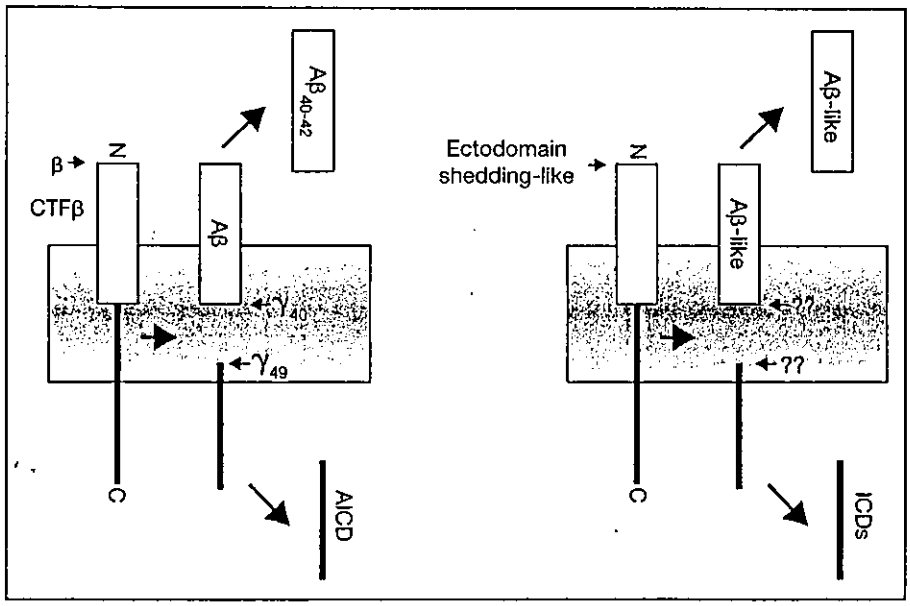

Fig. 3. Aβ-like peptides are secreted through signal transduction mediated by PS-dependent RIP.

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

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

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

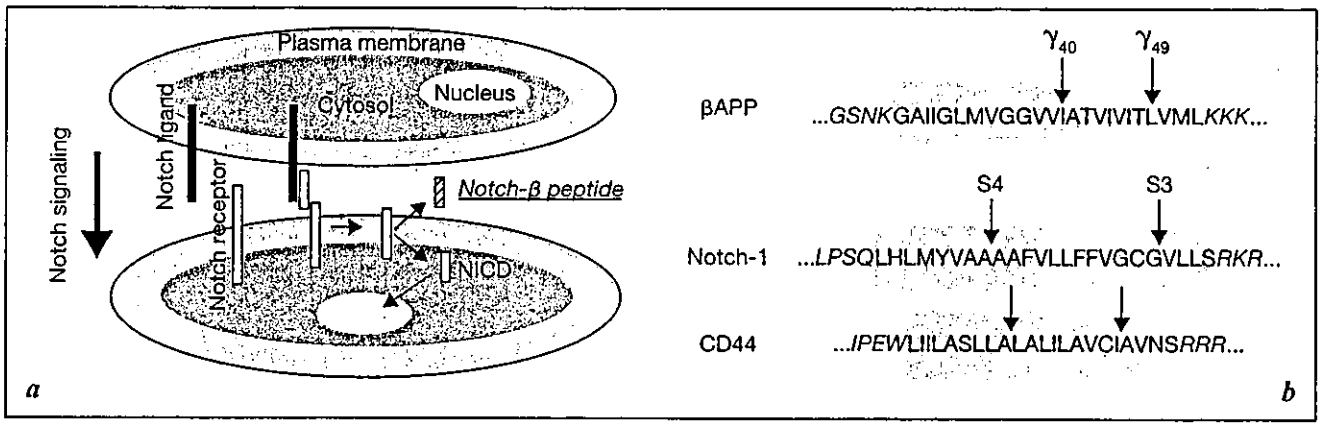

Fig. 4. ***a*** Notch signaling accompanies secretion of Notch-β peptide. By sequential endoproteolysis of Notch-1 shown in figures 2b, 3, an Aβ-like peptide, Nβ, is released. ***b*** PS-dependent intramembranous proteolysis, which we termed the 'dual cleavage' mechanism. Arrows indicate proteolytic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

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

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

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

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

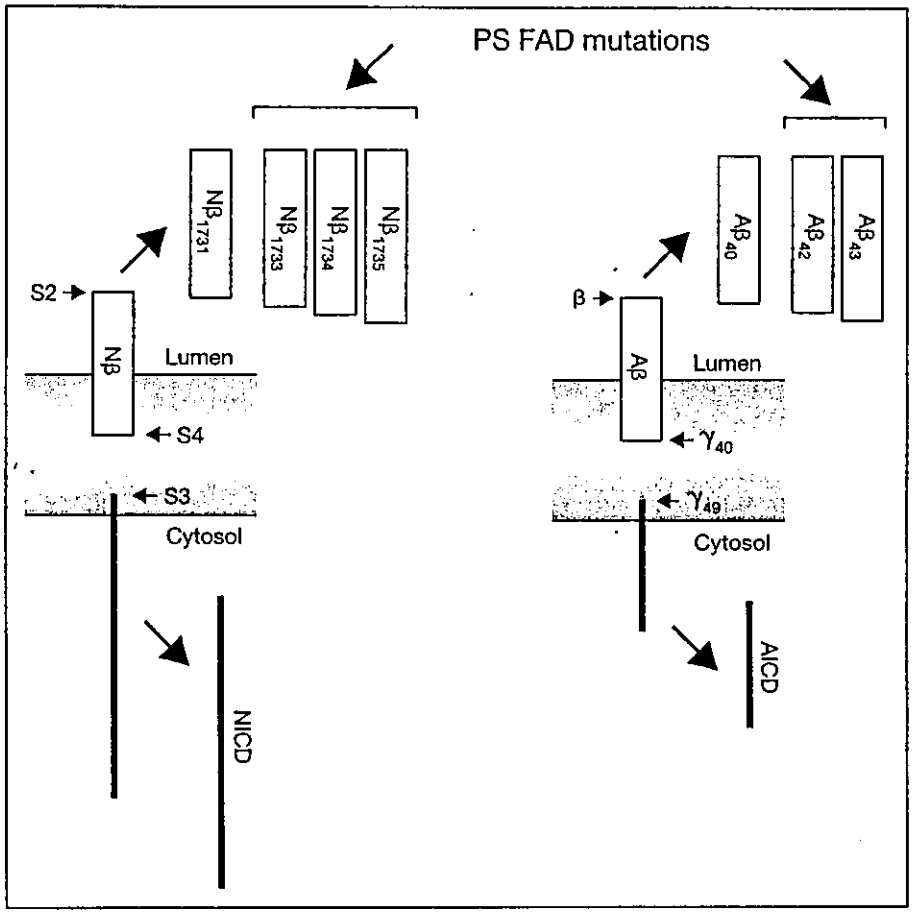

Fig. 5. Familial-AD-associated PS mutations affect γ-cleavage and elongate Nβ. These mutants showed a very similar effect on the precision of the γ-cleavage for the two distinct substrates. The magnitude of the effect, as analyzed so far, was not dependent on the substrates but on mutations of PS. In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

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

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

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

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Metals accelerate production of the aberrant splicing isoform of the presenilin-2

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Abstract

Oxidative stress is a major risk factor for Alzheimer's disease (AD) and other neurodegenerative disorders. Metals are known to be one of the factors that contribute to oxidative stress. Recently, we reported that the aberrant splicing isoform (PS2V) generated by skipping exon5 of the presenilin-2 (PS2) gene is a diagnostic feature of sporadic AD (SAD). PS2V is inducible by exposure of human neuroblastoma to hypoxia. We examined whether this aberrant splicing was caused by metal-induced oxidative stress, such as exposure to aluminum. As a result, we demonstrated that exposure to aluminum accelerated PS2V production induced by hypoxia. This acceleration of the production of PS2V to hypoxia was caused by chronic aluminum exposure, but was not related to the intracellular content of aluminum. HMGA1a is a mediator of PS2V production, and it was induced by aluminum as well as by hypoxia. Induction of HMGA1a was increased by chronic exposure to aluminum, and a nuclear extract containing HMGA1a bound to a specific sequence on exon5 of PS2 pre-mRNA, as reported previously. Finally, the acceleration of PS2V production induced by aluminum under hypoxic conditions reflected, but has not yet been directly shown to cause, vulnerability to endoplasmic reticulum stress. These results suggest that exposure to some metals can accelerate and enhance PS2V generation, and that hypoxia plus chronic exposure to metals may promote the development of AD.

Keywords: Alzheimer's disease, HMGA1a, hypoxia, metal, presenilin 2, splicing.

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Alzheimer's disease (AD) is a neurodegenerative disorder that is clinically characterized by progressive loss of memory and other cognitive abilities, and pathologically features severe neuronal loss, glial proliferation, extracellular senile plaques composed of amyloid-β protein (Aβ), and intraneuronal neurofibrillary tangles (reviewed in Selkoe 1994). In early onset forms of AD, some of these changes are caused by abnormalities of the amyloid precursor protein (APP) gene located on chromosome 21 as well as presenilin genes 1 and 2 located on chromosomes 14 and 1, respectively (Goate *et al.* 1991; Rogaev *et al.* 1995). Despite extensive research, however, little is known about the mechanisms that underlie sporadic AD, which accounts for over 90% of all cases of this disease.

Recently, we reported the preferential expression of a characteristic splicing variant of the PS2 gene that lacks exon 5 (an isoform termed PS2V) in sporadic AD brains, which was not caused by a mutation of the gene but possibly by a *trans*-acting factor (Sato *et al.* 1999). We showed that PS2V protein impaired the signaling pathway of the unfolded protein response (UPR), in a manner similar to familial AD-linked PS1 mutant proteins, and caused a significant increase in the

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Abbreviations used: Aβ, amyloid-β protein; AD, Alzheimer's disease; Al-maltol, aluminum maltolate; APP, amyloid precursor protein; HMGA1a, high mobility group A1a protein; PS2, presenilin-2; ROS, reactive oxygen species; SAD, sporadic Alzheimer's disease; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tm, tunicamycin; UPR, unfolded protein response.

production of Aβ protein (Katayama *et al.* 1999; Sato *et al.* 1999). Interestingly, PS2V-encoded aberrant proteins were observed in vulnerable pyramidal cells of the hippocampal CA1 region as well as in cells of the cerebral cortex in sporadic AD brains (Sato *et al.* 2001; Manabe *et al.* 2002). Furthermore, we found that PS2V could be induced in human neuroblastoma SK-N-SH cells by exposure to hypoxia and purified the responsible *trans*-acting factor based on its binding to an exon 5 fragment. As a result, high mobility group A1a protein (HMGA1a; formerly known as HMG-I) was identified as the factor (Manabe *et al.* 2003). The expression of HMGA1a was induced by hypoxia and subsequently led to the generation of PS2V (Manabe *et al.* 2003). In addition, the finding that antioxidants inhibited generation of the hypoxia-induced splice variant suggests that even at a low oxygen tension (8 Torr)(Sato *et al.* 1999), oxidative stress and the production of reactive oxygen species (ROS) may occur and with various deleterious effects (Borgem and Essig 1998). Indeed, recent studies have suggested that enhanced cellular oxidative stress may contribute to the progressive neurodegeneration that is observed in AD.

Metals play a major catalytic role in the production of free radicals, and attention has been paid to the role of various metals in AD, including iron, aluminum, copper, and zinc (Bush 2003). Iron is involved in the formation of free radicals, which have well-known deleterious effects, via the classical Fenton and Haber–Weiss reactions. Many observations have yielded evidence that iron metabolism is involved in AD (Loeffler *et al.* 1995; Kennard *et al.* 1996; Markesbery 1997; Smith *et al.* 1997). For example, the concentration of iron is elevated in the brains of AD patients, while iron, transferrin, and ferritin have all been found in senile plaque (Loeffler *et al.* 1995; Markesbery 1997). It was reported that copper can act as a catalyst in the production of ROS and it has been shown that the APP molecule contains a copper-binding site (Multhaup *et al.* 1996, 1998; Multhaup 1997; White *et al.* 2001). Moreover, copper is essential for the activity of enzymes, including cytochrome-c oxidase and Cu/Zn superoxide dismutase (Curtain *et al.* 2001; Linder and Hazegh-Azam 1996). Aluminum is thought to have a weaker effect on the formation of free radicals, but it has also been suggested as one of the causative factors involved in the onset of AD (Crapper *et al.* 1980; Pratico *et al.* 2002).

Here, we investigated whether aberrant splicing (production of PS2V protein) was caused by metal-induced oxidative stress, especially by exposure to aluminum at low concentrations that did not have a neurotoxic effect.

Materials and methods

Cell culture

Human neuroblastoma SK-N-SK cells were maintained in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA), as described previously (Manabe *et al.* 2003).

Preparation of aluminum maltolate

Aluminum maltolate (Al-maltol) was prepared from aluminum potassium sulfate dodecahydrate (Sigma-Aldrich/Katayama Chemical. Inc., Osaka, Japan) and maltol (3-hydroxy-2-methyl-4-pyron)(Sigma-Aldrich/Katayama Chemical. Inc., Osaka, Japan), as described previously (Finnegan *et al.* 1986).

Exposure to Al-maltol and hypoxia

Culture of cells and exposure to hypoxia were performed as described previously (Manabe *et al.* 2003). For short-term exposure to Al-maltol, SK-N-SH cells were treated with 2.5–250 μM Al-maltol for 21 h. For chronic exposure, cells were treated with 2.5 or 25 μM Al-maltol for 3 months. As a control, cells were exposed to maltol alone and untreated control cells were also prepared.

Aluminum quantitation

Cell pellets were freeze-dried, and then atomic absorbance spectrometry was used to measure the aluminum content (Meshitsuka and Inoue 1998; Meshitsuka *et al.* 1999)

Preparation of total RNA and RT-PCR

Preparation of total RNA from neuroblastoma SK-N-SH cells and RT-PCR were performed as described previously (Sato *et al.* 1999) with minor modifications. Reverse transcription was carried out using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). The PCR conditions were described previously (Sato *et al.* 1999). Experiments were repeated at least four times using separate cell cultures and consistent results were obtained in all cases.

Preparation of nuclear extracts and nuclear fraction

Nuclear extracts and nuclear fractions were prepared from cultured cell lines as described elsewhere (Schreiber *et al.* 1989) with minor modifications (Yoneda *et al.* 1999; Manabe *et al.* 2000).

Preparation of RNA probes

RNA probes [including the no.5 probe (41nt)] were prepared by *in vitro* transcription with DNA template constructs (Manabe *et al.* 2003) in pcDNA3 vectors (Invitrogen, Carlsbad, CA, USA), as described previously (Manabe *et al.* 2003). In brief, the template plasmid (linearized with EcoRI) was incubated for 1 h at 37°C in a transcription reaction mixture (20 μL) containing 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.25 mM UTP, [^{35}S]UTPαS, 40 units of RNase inhibitor (TOYOBO, Tokyo, Japan), and 20 units of T7 RNA polymerase with the reaction buffer provided by the manufacturer (Promega).

UV cross-linking assays

The UV cross-linking assay was also described previously (Manabe *et al.* 2003). Briefly, an aliquot of nuclear extract (estimated protein content: 5 μg) was incubated for 30 min at 25°C in 25 μL of incubation buffer [12 mM HEPES–NaOH (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride] with 10 μg of tRNA and a ^{35}S-labeled RNA probe (1 μg). This reaction mixture

was irradiated with UV light (254 nm, 60 W) for 15 min at room temperature. Free probes were digested at 25°C for 30 min with 10 μg of RNase A, followed by the addition of a four-fold volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample dye mixture [10 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol]. After separation by SDS–PAGE on 15% polyacrylamide gel, the gel was fixed, dried, and analyzed with a Bio Imaging Analyzer (BAS-5000; Fujifilm Medical Systems). Each experiment was repeated at least three times using separate cell cultures and consistent results were obtained in all cases.

Immunoblotting assay

Immunoblotting was performed as described previously (Manabe *et al.* 2001a, 2001b) with minor modifications. Anti-HMGA1 [HMG-I(Y) (N-19)] goat polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for this assay.

Cell death assay

To estimate the vulnerability of cells, cell death was assessed on the basis of morphological changes observed by phase contrast microscopy or nuclear changes detected by fluorescence microscopy after costaining the cells with 10 μM Hoechst 33342 and 10 μM propidium iodide. That is, nuclear fragmentation was detected by Hoechst-positive staining and nuclear membrane collapse was detected by propidium iodide-positive staining. We determined dead cells by the above double positive. The staining was measured independently in 10 fields and at least 500 cells were counted, and the data was expressed as the mean ± SEM for three independent experiments. The percentage (%) of dead cells was calculated after each treatment relative to control cultures.

Results

Transient exposure to various metals

First, we examined the production of PS2V when SK-N-SH neuroblastoma cells were exposed to various metals. PS2V was produced after exposure of the cells to $FeCl_2$, $FeCl_3$, $ZnCl_2$, $CuCl_2$, $CuSO_4$, $AlCl_3$, and Al-maltol (Fig. 1a). However, the level of PS2V production was not consistent between experiments using a given concentration of metal, except in the case of aluminum, since addition of $AlCl_3$ and Al-maltol resulted in the reproducible production of PS2V (Fig. 1a). Accordingly, we performed the following experiments using Al-maltol as the test metal.

As shown in Fig. 1, expression of PS2V was increased by about 12-fold after exposure to hypoxia for 24 h (Fig. 1b, control), consistent with our previous report. Exposure to Al-maltol at a concentration of 25 or 250 μM also caused the production of PS2V without any exposure to hypoxia. Furthermore, exposure to Al-maltol promoted the production of PS2V at 12 h after hypoxia even when PS2V was not induced by hypoxia alone (Fig. 1b, squares). Although the promotion of PS2V production was also seen at 24 h after hypoxia followed by exposure to Al-maltol at a concentration of 25 μM, Al-maltol at a concentration of 250 μM caused a decrease to control levels of PS2V thereafter (Fig. 1b).

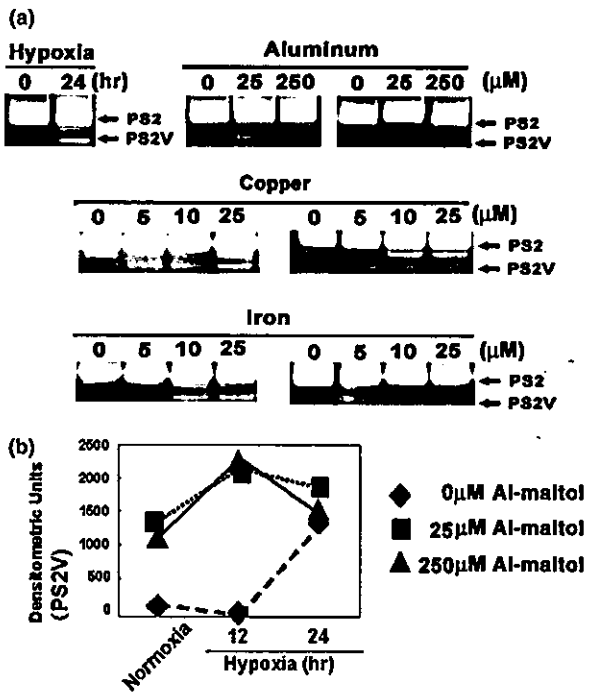

Fig. 1 Effect of metals on the expression of PS2V in SK-N-SH cells. (a) RT-PCR-amplified products were separated on a polyacrylamide gel and visualized by ethidium bromide staining. Arrows indicate the positions of the normal PS2 transcript and the aberrant PS2V transcript. Total RNA was extracted from the cells exposed to indicated metals (Al-mal, aluminum; $CuCl_2$, copper; $FeCl_2$, iron) or hypoxia-exposed cells. Each column shows the independent experimental data. (b) Effect of short-term incubation with Al-maltol on hypoxia-induced expression of PS2V. Cells were incubated without Al-maltol (rhomboids) or with 25 μM (■) or 250 μM (▲) Al-maltol for 24 h, followed by hypoxic stimulation. The cells were harvested at 0–24 h after hypoxic stimulation and total RNA was prepared for RT-PCR of PS2V. Quantitative data were obtained by densitometry of the band corresponding to the molecular weight of PS2V (shown as a percentage of control).

Chronic exposure to a low concentration of Al-maltol

Next, we examined the effect of chronic exposure to a low concentration of Al-maltol on the hypoxia-induced expression of PS2V in SK-N-SH cells. As shown in Fig. 2, there was no significant expression of PS2V when SK-N-SH cells were exposed to 2.5 μM or 25 μM Al-maltol for 3 months under normoxic conditions (Fig. 2, normoxia). On the other hand, hypoxia of 0.5 h or more caused a significant increase of PS2V expression in SK-N-SH cells exposed to 2.5 μM or 25 μM Al-maltol for 3 months. This potentiation by hypoxia was maximal over a period of 4 h and persisted for at least 8 h (Fig. 2). However, aluminum

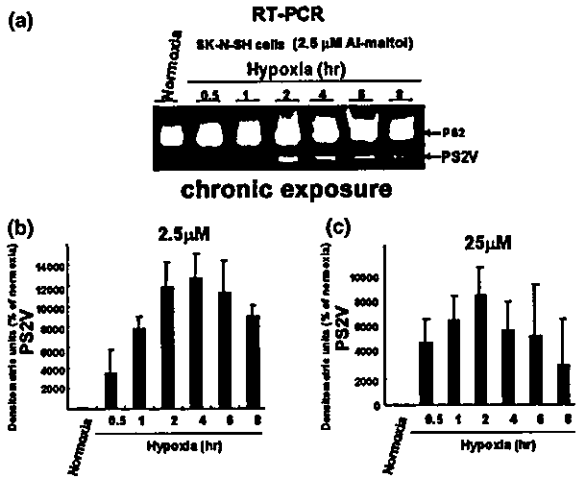

Fig. 2 Effect of chronic incubation with low concentrations of Al-maltol on hypoxia-induced expression of PS2V in SK-N-SH cells. SK-N-SH cells were cultured for three months in α-MEM (10% FCS) containing 2.5 or 25 μM Al-maltol, followed by hypoxic stimulation. Cells were harvested at 0–8 h after hypoxia and total RNA was prepared for RT-PCR of PS2V. (a) Representative data was shown (2.5 μM). RT-PCR-amplified products were separated on a polyacrylamide gel and visualized by ethidium bromide staining. Arrows indicate the positions of the normal PS2 transcript and the aberrant PS2V transcript. (b and c) Quantitative data were obtained by densitometry of the band corresponding to the molecular weight of PS2V (shown as a percentage of control).

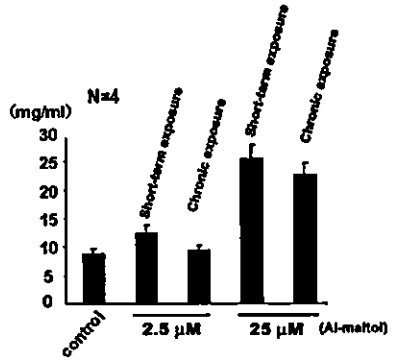

Fig. 3 Intracellular content of aluminum. Cells underwent short- or long-term incubation with Al-maltol. The treated cells were harvested and freeze-dried, followed by measurement of the intracellular aluminum content by atomic absorbance spectrometry. The data were obtained from four separate experiments (mean ± SE).

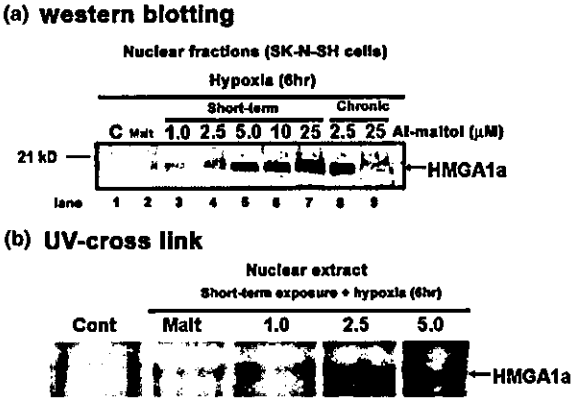

Fig. 4 Effect of Al-maltol on hypoxia-induced HMGA1a expression in SK-N-SH cells. SK-N-SH cells were incubated with Al-maltol for 6 h or 3 months. The treated cells were then exposed to hypoxia for 6 h, followed by preparation of either nuclear fractions (a) or nuclear extracts (b). (a) Nuclear fractions were subjected to SDS–PAGE (15%) after immunoblotting using an anti-HMGA1a antibody. The arrow indicates the position of HMGA1a protein (~18 kDa). (b) Nuclear extracts were analyzed by the UV cross-linking assay with no. 5 probe and subjected to SDS–PAGE (15% gel). The no. 5 probe is an RNA probe that has the sequence which HMGA1a specifically recognizes (Manabe *et al.* 2003). The arrow indicates the position of HMGA1a protein.

concentration dependence of the induction of PS2V expression was not observed.

Intracellular aluminum content of SK-N-SH cells exposed to Al-maltol for 3 months

Next we investigated the accumulation of aluminum after short-term and chronic exposure of SK-N-SH cells to a low concentration Al-maltol by using the atomic absorbance spectrometry. As a result, intracellular accumulation of aluminum increased in an Al-maltol concentration-dependent manner after both brief and chronic exposure (Fig. 3). However, aluminum accumulation was not dependent on the duration of exposure in this experiment. Therefore, our findings raised the possibility that the acceleration by chronic low concentration of aluminum depends on the incubation period, but is not related to intracellular accumulation of aluminum during chronic exposure of SK-N-SH cells.

Induction of HMGA1a by Al-maltol incubation

It had been demonstrated that PS2V is generated by HMGA1a in SK-N-SH cells (Manabe *et al.* 2003). Consequently, we investigated the effect of Al-maltol on expression of hypoxia-induced HMGA1a in SK-N-SH cells. Significant expression of HMGA1a protein was not observed in SK-N-SH cells after hypoxic stimulation for 6 h (Fig. 4a, lane 1). However, expression of HMGA1a protein was increased in Al-maltol-treated cells (short-term exposure) after 6 h of hypoxia, compared with control cells and the increase was dependent on the Al-maltol concentration (Fig. 4a, lanes 2–7).

At this time, the binding of HMGA1a to its recognition RNA sequence (no. 5 RNA probe; length 41nt) was significantly increased in a dose-dependent manner (Fig. 4b).

Strong HMGA1a immunoreactivity was observed in nuclear fractions isolated from chronic exposed Al-maltol-treated SK-N-SH cells after 6 h of hypoxia (Fig. 4a, lanes 8 and 9).

Aluminum promotes cell death by endoplasmic reticulum (ER) stress

We previously demonstrated that cells expressing PS2V or exposed to hypoxia exhibited increased vulnerability to various ER stresses, the mechanism of which was related to the down-regulation of UPR signaling (Sato *et al.* 2001; Manabe *et al.* 2003). To confirm that the increase of PS2V production induced by Al-maltol under hypoxic conditions reflected an increase in susceptibility to ER stress, we investigated cell death on the basis of morphological changes (Fig. 5). Cell death induced by tunicamycin (Tm), which is a major ER stress inducer, was significantly promoted by hypoxia, consistent with previous observations (Manabe *et al.* 2003). This promotion was accelerated by chronic exposure to Al-maltol at a concentration of 2.5 μM (Fig. 5b) or 25 μM (data not shown). From 6 to 12 h after Tm treatment, the extent of cell death was different between Al-maltol-treated and untreated (control) cultures. On the other hand, under the normoxic condition, the extent of cell death was unaffected by short-term incubation with Al-maltol at a concentration of 25 or 1000 μM, nor by chronic exposure to 2.5 or 25 μM (Fig. 5a). Consequently, it was clarified that cells exposed to Al-maltol were more sensitive to ER stress, as was observed for cells expressing PS2V. Finally, we compared chronic Al-maltol treated group with control group on the ratio of extent of cell death by Tm plus hypoxia treatment to the extent by Tm alone treatment (Fig. 5c). In every indicated time, the ratio by Tm + Hypo was higher than the ratio by Tm alone. This result suggested that effect of chronic Al-maltol exposure is acceleration to hypoxia (means production of PS2V) but not enhancement to Tm toxicity.

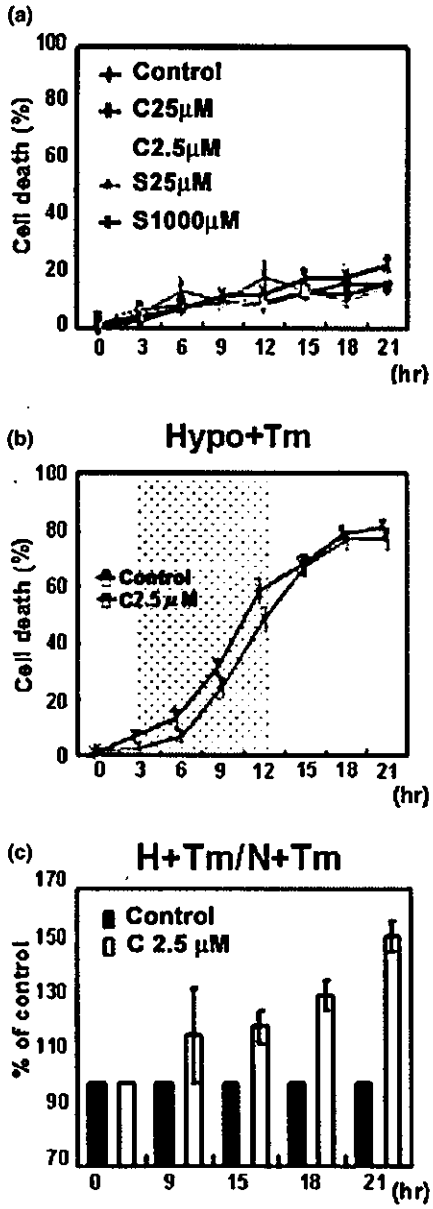

Fig. 5 Effect of Al-maltol on cell death associated with PS2V expression in SK-N-SH cells exposed to ER stress. (a) Viability of SK-N-SH cells with chronic (C) or short-termed (S) Al-maltol-treatment under normoxia for 21 h, followed by assay of morphological changes. (b) The effects of Al-maltol treatment on SK-N-SH cell viability under hypoxia exposure plus tunicamycin (Tm) stress (ER stress) were evaluated by counting of morphological changes in indicated time. The cells were treated with 0.5 μg/mL Tm and exposed to hypoxia. Data are shown as a percentage of the control value (0 h) (mean ± SE). (c) Comparison between chronic Al-maltol treated group (C2.5) and control group (control) on the ratio (cell death score by Tm treatment plus hypoxia exposure to the score by Tm treatment alone: Hypoxia + Tm/Normoxia + Tm). Data are shown as a percentage of the control group ratio.

Discussion

We examined the effects of transient exposure to various metal salts ($FeCl_2$, $FeCl_3$, $CuCl_2$, $CuSO_4$, $ZnCl_2$, $AlCl_3$, and Al-maltol). However, except in the case of aluminum, the same level of PS2V production was not detected between experiments using the same concentration of each metal. In contrast, addition of $AlCl_3$ and Al-maltol reproducibly caused the production of PS2V. Abnormalities of iron, copper and zinc have been reported as risk factors for the onset of AD. However, it is difficult to monitor the intracellular content of these metals and distinguish endogenous from exogenous sources because each of these metals is required by living cells. On the contrary, aluminum is not a necessary metal for cells, so it is relatively easy to estimate the effect of aluminum treatment by measuring its content in the target cells. In the present study, we therefore focused on the influence of aluminum as a proxy for various metals.

Production of PS2V was slightly induced by 25 or 250 μM Al-maltol without hypoxia. Under hypoxic conditions, however, PS2V was more strongly and rapidly expressed after exposure to Al-maltol treatment. At 24 h after hypoxia, it was interesting that 250 μM Al-maltol induced less PS2V than 25 μM. This suggested that excessive oxidative stress did not induce production of PS2V, consistent with our previous report that exposure to H_2O_2 did not induce PS2V (Sato *et al.* 1999).

Chronic Al exposure accelerates PS2V production

Exposure to a low (2.5 μM) or moderate (25 μM) concentration of Al for 3 months caused an increase in the production of PS2V after hypoxic exposure, but there was no difference in the increment of PS2V production between both concentrations (Fig. 3). To determine whether the increment of PS2V production was based on the accumulation of aluminum or chronic stimulation, we measured the intracellular content of aluminum by atomic absorbance spectrometry. Interestingly, there was no further increase of intracellular aluminum in SK-N-SH cells by long-term exposure to Al-maltol despite an increase of the cellular aluminum content according to the concentration of Al-maltol. This finding suggests that the acceleration of PS2V production was caused by chronic aluminum exposure, but did not depend on the aluminum concentration.

HMGA1a expression is induced by Al-maltol

Recently, we reported that HMGA1a is a mediator of aberrant PS2 pre-mRNA splicing and the production of a deleterious PS2V protein (Manabe *et al.* 2003). In that experiment, HMGA1a was inducible by hypoxia only in a neuronal cell line. In the present study, HMGA1a was dose-dependently induced by Al-maltol (Fig. 4a). The induction of HMGA1a was increased by long-term exposure of SK-N-SH cells to Al-maltol, and the nuclear extract containing HMGA1a bound to a specific sequence on PS2 pre-mRNA exon 5, which was located upstream of the 5′ splicing site (Fig. 4b). These results indicate that production of PS2V stimulated by Al-maltol occurred by an equivalent mechanism to production due to hypoxia.

Neuronal cell death and aluminum

Chronic aluminum neurotoxicity is well known on the basis of the fact that dialysis dementia occurs in adults and children with renal insufficiency who are treated with aluminum-contaminated dialysate solutions or oral phosphate-binding agents that contain aluminum (Alfrey *et al.* 1976; American Academy of Pediatrics Committee on Nutrition 1986). However, most previous studies about the relationship between aluminum and AD have assessed the effects of high doses of aluminum. For example, transient or chronic administration of 60–8300 mg/kg p.o., 1–60 mg i.v., or 0.1–5 mg i.c.v. *in vivo* and 100–1000 μM *in vitro* were tested in the previous studies (Gawlick *et al.* 1987; Martyn *et al.* 1989; ATSDR 1999). On the contrary, we used low concentrations of 2.5–25 μM *in vitro*, and these levels are known not to show neurotoxicity (Alfrey *et al.* 1976). Consequently, there are differences between high-dose aluminum toxicity in dialysis dementia and the effect of low concentrations of aluminum shown in the present study. In spite of the low concentration, cell death induced by ER stress and/or by hypoxia was promoted by chronic exposure to aluminum. The reasons are unclear, but it is thought that oxidative stress was at least partly involved in this promotion of cell death. Although aluminum salts do not stimulate peroxidation per se, these salts greatly accelerate peroxidation induced by iron(II) salts (Savory *et al.* 1999). Also, our previous study showed that pretreatment of SK-N-SH cells with antioxidants before exposure to hypoxia could completely suppress the production of PS2V (Sato *et al.* 1999). Taken together, our data suggest that the combined stresses of hypoxia and ROS generation by metals including aluminum are necessary to trigger an increase of PS2V and consequent vulnerability to ER stress.

In conclusion, our findings suggest that some metals at low concentrations can accelerate and enhance PS2V generation. Accordingly, exposure to hypoxia plus the long-term intake of such metals at low concentrations may promote the development of AD. Consequently, such hypothesis may provide to shed light on the pathogenesis and development of sporadic cases of AD.

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