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Cholesterol-dependent generation of a unique amyloid β-protein from apically missorted amyloid precursor protein in MDCK cells

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Abstract

To investigate the implications of altered sorting of the β-amyloid precursor protein (βAPP) in the abnormal generation of amyloid β-protein (Aβ), we characterized Aβ secreted from Madin-Darby canine kidney (MDCK) cells which had been stably transfected with a cDNA encoding the human β-amyloid precursor protein (βAPP695) with a 42 amino acid residue truncation at the carboxyl terminus (ΔC). In ΔC MDCK cells, the intracellular sorting of βAPP is substantially altered to the apical surface. We detected an accumulation of a unique Aβ species in the apical compartment of ΔC MDCK cell cultures. This unique Aβ was immunoprecipitated with 4G8 (a monoclonal antibody specific for Aβ17–24) and detected as a smear on Western blots, but was not immunoprecipitated with BAN50 (a monoclonal antibody raised against Aβ1–16). Interestingly, however, this Aβ species was readily immunoprecipitated with BAN50 upon treatment with formic acid. Furthermore, incubation of the ΔC MDCK cells with compactin, an inhibitor of de novo cholesterol synthesis, or with filipin, a cholesterol-binding drug, resulted in marked changes in the characteristics of this Aβ species as follows: first, the Aβ was not observed as a smear on Western blots and second, the Aβ was immunoprecipitated with BAN50. The present results strongly suggest that an Aβ with unique molecular characteristics is generated from the missorted βAPP in vivo in a cholesterol-dependent manner.

Keywords: Alzheimer's disease; Amyloid β-protein; Madin-Darby canine kidney cell; Cell polarity; Cholesterol

1. Introduction

The abnormal accumulation of amyloid β-protein (Aβ), a proteolytic cleavage product of β-amyloid precursor protein (βAPP), a large membrane-spanning glycoprotein [1], is an invariable neuropathological feature of Alzheimer's disease (AD). Several lines of evidence indicate that abnormal generation of Aβ due to altered processing of βAPP is a fundamental step in the development of AD. The cellular mechanisms underlying the generation and secretion of Aβ are still far from achieving a complete understanding, although previous studies have revealed that Aβ is generated in acidic intracellular organelles such as endosomes following internalization of βAPP [2,3] and/or in the endoplasmic reticulum [4]. It is known that Aβ is cleaved from βAPP at the amino and carboxyl termini of Aβ by β- and γ-secretases, respectively (Aβ-generating pathway). βAPP is also cleaved

Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; βAPP, amyloid precursor protein; CLD, caveolae-like domain; EIA, enzyme immunoassay; MDCK cell, Madin-Darby canine kidney cell

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by α-secretase within the Aβ sequence, liberating the soluble derivative of βAPP (APPs) (APPs-secreting pathway). In the latter case, the generation of Aβ is precluded. Previous studies have demonstrated that the activation of protein kinase C causes a shift from the Aβ-generating pathway to the APPs-secreting pathway (for review, see [5]). It was recently reported that intracellular and secreted Aβ species are generated via distinct βAPP processing pathways [6]. Thus, intracellular sorting of βAPP is likely to be one of the factors determining the generation of Aβ.

The aim of this study was to investigate the role of altered sorting of βAPP in the abnormal generation of Aβ. We characterized the Aβ secreted from Madin-Darby canine kidney (MDCK) cells which have been extensively studied in terms of cell polarity [7]. It is known that, in MDCK cells, βAPP undergoes highly polarized sorting principally to the basolateral surface [8]. As previously reported [8,9], the majority of Aβ is basolaterally secreted from MDCK cells. We extended this line of experimentation to the study of MDCK cells which had been transfected with βAPP cDNA encoding a 42 amino acid carboxyl-terminal truncated βAPP (ΔC MDCK cells), in which the intracellular sorting of βAPP is substantially altered to the apical surface [9].

Recently, much attention has been focused on the physiological roles of cholesterol and glycosphingolipids which form specific lipid microdomains destined for the apical surfaces of epithelial cells (for review, see [10]). Regarding the pathological implications of these lipid microdomains, it is noteworthy that such microdomains in neurons (caveolae-like domains, CLDs) are likely to be the site of conversion of a normal cellular form (PrP^{c}) of prion protein to a pathological, amyloidogenic scrapie form (PrP^{sc}) with a high proportion of β-sheet structures, which causes fatal neurodegenerative diseases [11,12]; cholesterol depletion inhibits the formation of the scrapie form [11]. Thus, it is intriguing to investigate the molecular characteristics of Aβ which is generated in membranes containing abundant cholesterol and glycosphingolipids. An advantage of using ΔC MDCK cells is that we could determine the effect of the cholesterol and/or glycosphingolipid which are abundant in the apical membranes [13,14], on the generation of Aβ.

In this study, we detected a unique form of Aβ species in the medium of the apical compartment of the ΔC MDCK cell cultures. The most striking result that emerges from this study is that the generation of this unique Aβ is dependent on the level of intracellular cholesterol. These results may provide a new insight into the pathological implications of the altered sorting of βAPP in the abnormal generation of Aβ in the brains of patients with AD.

2. Material and methods

2.1. MDCK cell culture

MDCK cells were cultured as previously described [8,9,15]. Dulbecco's modified Eagle's medium (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS) was used as the culture medium. We plated $2.5–3.0\times10^{5}$ MDCK cells transfected with human βAPP695 cDNA onto 24 mm Transwell filters (Costar) and then cultured these cells on the filters for 3 days. To determine the integrity of the cell monolayers that grew on the filters, measurement of the electrical resistance between apical and basolateral compartments of the MDCK cell cultures was performed by immersing electrodes in each compartment (according to a protocol provided by World Precision Instruments). Complete integrity of the cell monolayers was confirmed by the absence of the leakage of Aβ which had been added to the basolateral compartment, into the apical compartment. The media of the MDCK cell cultures were changed 3 days after plating, and the cells were then cultured for a further 24 h. Finally, the media in both compartments were collected for immunochemical analysis. The procedures for synthesis and transfection of βAPP cDNA were as previously described [8,9]. Briefly, cDNA encoding the full-length and 42 amino acid carboxyl-terminal truncated forms of human βAPP695 were generated by PCR using an oligonucleotide designed to introduce stop codons at the corresponding positions. Stable transfection of MDCK cells and the selection of single cell clones were performed as previously described [8].

2.2. Antibodies

The anti-Aβ monoclonal antibodies used in this

study were BAN50 (raised against Aβ1–16), BA27 (specific for Aβ40), BC05 (specific for Aβ42 or Aβ43) and 4G8 (specific for Aβ17–24), whose characteristics have been previously described [16–18].

2.3. Enzyme immunoassay

The two-site enzyme immunoassay (EIA) for Aβ was performed essentially as previously described [16,17]. Briefly, 100 μl of the media of the apical and basolateral compartments of the MDCK cell cultures were diluted with 400 μl of buffer EC (20 mM phosphate buffer (pH 7.0), 0.4 M NaCl, 2 mM EDTA, 10% Block Ace (Dai-nippon, Tokyo, Japan), 0.2% bovine serum albumin and 0.05% NaN_3), and 100 μl of the mixture were subjected to the two-site EIA. The BAN50 and 4G8, used as capture antibodies, were coated onto multiwell plates, and BC05 and BA27 were used as detector antibodies following their conjugation with horseradish peroxidase. An appropriate amount of synthetic Aβ40 or Aβ42 (Bachem California, CA, USA), was applied, for the construction of a standard curve, to a BAN50-coated multiwell plate which was incubated at 4°C overnight. After rinsing with phosphate-buffered saline (PBS), loaded wells were reacted with appropriately diluted horseradish peroxidase-conjugated BA27 or BC05 at 4°C overnight. Bound enzyme activities were measured using the TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Labs., Gaithersburg, MD, USA).

2.4. Immunoprecipitation and Western blotting

Aliquots (1.5 ml) of the media from the cultures of transfected MDCK cells, 24 h after reaching complete integrity of the cell monolayer, were incubated with BAN50 (5.8 μg) at 4°C overnight for Aβ detection. The mixtures were then incubated with protein G Sepharose at 4°C for 3 h, and centrifuged. In the experiments for Aβ detection, the resultant supernatants were subsequently incubated with 4G8 (1.5 μg) at 4°C overnight. The pellets were washed thoroughly with Tris-saline buffer, and subjected to protein solubilization by boiling in Laemmli buffer containing 8 M urea for 5 min. The solubilized proteins were separated on 4–20% gradient Tris-tricine SDS-polyacrylamide gels (Multigel 4/20, Daiichi Chemical, Japan). Western blotting was performed according to the method of Ida et al. [19]. Briefly, the proteins were transferred onto a 0.22 μm nitrocellulose membrane (Micron Separation, MA, USA) at 380 mA for 45 min using a semi-dry blotting apparatus; the blots were heated in boiling PBS (8.1 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, 137 mM sodium chloride, and 2.7 mM potassium chloride, pH 7.4) for 5 min, and then blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 30 min at room temperature (RT). After rinsing, the blots were incubated with BA27 (1 μg/ml) for Aβ detection. The antibodies were diluted with 0.025% bovine serum albumin (BSA)-containing PBS-T. The blots were washed 4 times at RT with PBS-T, and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Gibco, BRL) as a secondary antibody, diluted with 0.025% BSA-containing PBS-T, at 4°C overnight. The blots were again washed as described above, and the bound-enzyme activities were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK).

2.5. Formic acid treatment

The 4G8 immunoprecipitates from 1.5 ml aliquots of the media from the apical and basolateral compartments of the ΔC MDCK cell cultures were incubated in 200 μl of highly concentrated formic acid at RT for 30 min. The mixtures were then centrifuged at $15\,000 \times g$ for 15 min. The supernatants were vacuum-dried, and the residual pellets solubilized by boiling for 5 min in 25 μl of Laemmli buffer containing 8 M urea. The solubilized Aβ was diluted with 975 μl of Tris-saline buffer, and incubated with BAN50 (3.8 μg/ml) at 4°C overnight for reimmunoprecipitation. Western blotting was performed as described above.

2.6. Inhibition of de novo cholesterol synthesis

To inhibit de novo cholesterol synthesis, MDCK cells were incubated for 2 h with compactin (Sigma), a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity at concentrations ranging from 100 nM to 5000 nM and then

exposed to 2 μCi of [^{14}C]acetate. After a further 2 h, all cells were harvested, and the concentration of newly synthesized cholesterol was quantitated by determining the amount of radioactivity incorporated into free cholesterol separated by thin layer chromatography, using a Bio-imaging Analyzer System-2500 Mac (Fuji Photo Film, Japan). To investigate the effect of the inhibition of de novo cholesterol synthesis on the secretion of Aβ, ΔC MDCK cells were pretreated with compactin for 90 min before the media were changed to fresh ones. The cells were cultured for a further 3 days with compactin, and then 1.5 ml aliquots of the media from the apical and basolateral compartments of the culture were immunoprecipitated as described above.

2.7. Treatment with filipin

MDCK cells were cultured in media containing filipin (Sigma), a polyene antibiotic which specifically binds to cholesterol, at concentrations of 0.1 μg/ml and 0.3 μg/ml for 90 min before the media were changed to fresh ones. The cells were cultured for a further 3 days with filipin, and then 1.5 ml aliquots of the media from the apical and basolateral compartments of the culture were immunoprecipitated as described above.

2.8. Densitoscanning and statistical analysis

The intensity of the bands corresponding to Aβ was determined densitometrically. The relative intensity values of the Aβ detected in the medium of apical compartment to those of the Aβ in the medium of basolateral compartment were statistically analyzed using Student's *t*-test.

3. Results

3.1. MDCK cell culture system

The MDCK cells ($2.5–3.0\times10^5$) were plated onto a 24 mm Transwell filter and then cultured for 3 days. The growth of the cells was indirectly monitored by phase-contrast microscopic observation of a sister culture plated onto a 35 mm culture dish at the same cell density. The cultured cells appeared confluent at 48 h after plating, and the electrical resistance between the apical and the basolateral compartments decreased slightly following a peak at 48 h, and then reached a plateau (Fig. 1). For confirmation of the integrity of the cell monolayer, the amount of the leaked Aβ was determined by EIA; no Aβ leakage was detected at 72 h after plating (Fig. 1).

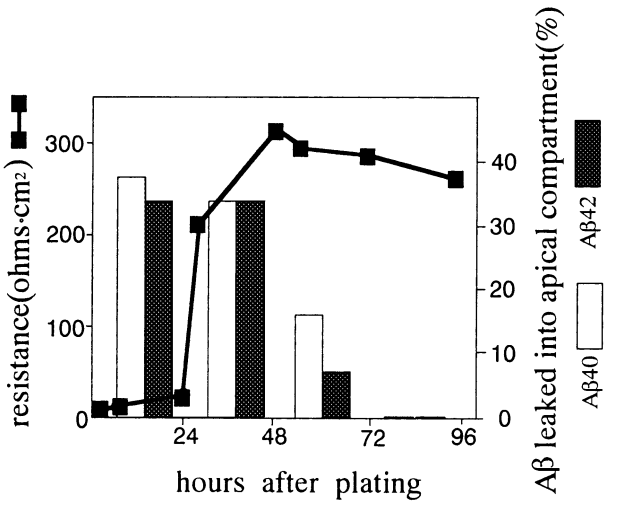

Fig. 1. Measurement of transmembrane electrical resistance, and the percentage of Aβ leaked in MDCK cell cultures. MDCK cells transfected with human βAPP695 cDNA were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The grown cells were replated at semi-confluent density onto 24 mm Transwell filters (Costar) ($2.5–3.0\times10^5$ cells/well). The cells on the filters were then incubated with 1.5 ml of medium in the apical compartment, 2.5 ml in the basolateral compartment, and grown for a further 3 days. Transmembrane electrical resistance was measured with a device (EVOM) from World Precision Instruments. Synthetic Aβ (500 fmol of Aβ40 or Aβ42) was added to the basolateral compartment. The levels of Aβ which leaked into the apical compartment from the basolateral compartment were determined by EIA as described in Section 2. BAN50-coated multiwell plates were used.

3.2. Polarized secretion of Aβ from wild-type and ΔC MDCK cells

To investigate the polarized secretion of Aβ from ΔC MDCK cells, we employed a procedure of immunoprecipitation of Aβ with BAN50. Blots of the immunoprecipitates were reacted with BA27. We detected the polarized secretion of Aβ40 from the ΔC MDCK cells predominantly into the basolateral compartment, essentially as for the case of the

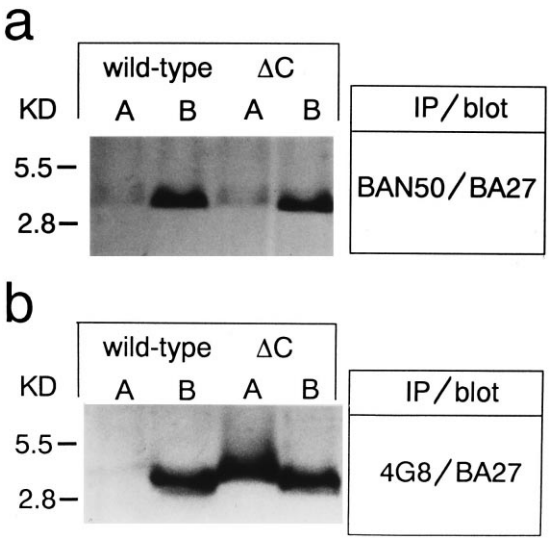

Fig. 2. Polarized secretion of Aβ40 from MDCK cells transfected with human βAPP695 cDNA. (a) Immunoprecipitation of Aβ40 secreted into the media of the apical and basolateral compartments with BAN50 (a monoclonal antibody raised against Aβ1–16). (b) Subsequent immunoprecipitation of Aβ40 in the media which had been preincubated with BAN50, with 4G8 (a monoclonal antibody specific for Aβ17–24). The blots of the immunoprecipitates were reacted with BA27 (a monoclonal antibody specific for Aβ40). Wild-type and ΔC indicate the MDCK cells transfected with cDNA encoding the full-length and carboxyl-terminal truncated human βAPP, respectively. (A) apical compartment; (B) basolateral compartment.

wild-type MDCK cells (Fig. 2a). These results strongly suggest that a fraction of Aβ40 molecules is generated intracellularly, before βAPP is sorted to the cell surface. Regarding the generation of Aβ from the apically transported βAPP in the ΔC MDCK cells, we considered the possibility that amino-terminal truncated Aβ, which is not recognized by BAN50, was secreted. To test this possibility, the monoclonal antibody 4G8 was used for the subsequent immunoprecipitation of Aβ in the medium from the apical compartment of ΔC MDCK cell cultures, which had been preincubated with BAN50. We detected strong BA27 immunoreactivity in the medium of the apical compartment of ΔC MDCK cell cultures on a Western blot (Fig. 2b). Interestingly, the Aβ was detected as a smear on the blot, and its electrophoretic mobility was lower than that of Aβ secreted into the basolateral compartment of the wild-type and the ΔC MDCK cell cultures (Fig. 2b).

3.3. *Incubation of synthetic Aβ in the media of MDCK cells*

To investigate whether the unique molecular characteristics of the Aβ, including its appearance as a smear and its retarded mobility in SDS-polyacrylamide gel electrophoresis, are caused by the presence of lipids coprecipitated from the media, we performed immunoprecipitation of synthetic Aβ1–40 which had been incubated in the media of the apical and basolateral compartments of non-transfected MDCK cells. As shown in Fig. 3, smearing of the Aβ was not observed on the blots. Thus, it appears likely that the unique Aβ in the media of the apical compartment of ΔC MDCK cells is generated prior to its secretion.

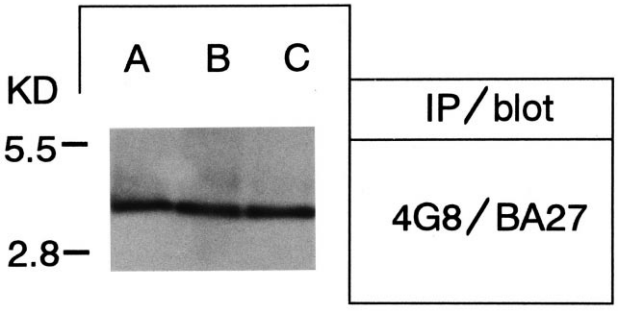

Fig. 3. Western blot of immunoprecipitated synthetic Aβ1–40, which had been incubated in the media of the apical (A) or basolateral (B) compartments of non-transfected MDCK cells, or in fresh media (C). Two hundred pg of synthetic Aβ1–40 were incubated in the media for 24 h and immunoprecipitated with 4G8. The blot of the immunoprecipitates was reacted with BA27.

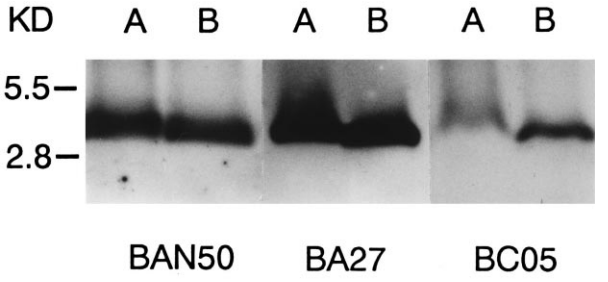

Fig. 4. Characterization of Aβ in the apical compartment of ΔC MDCK cells. The Aβ immunoprecipitated with 4G8, in the apical compartment of ΔC MDCK cells, was analyzed by Western blotting using various anti-Aβ monoclonal antibodies. Note that the Aβ was recognized by BAN50, which was raised against Aβ1–16, and that both Aβ40 and Aβ42 in the apical compartments appear as a smear on immunoblotting.

3.4. Characterization of Aβ immunoprecipitated with 4G8

To characterize the Aβ which is immunoprecipitated with 4G8 from the medium in the apical compartment of ΔC MDCK cell cultures, the immunoprecipitated Aβ was analyzed by Western blotting using various anti-Aβ monoclonal antibodies. On the blot, the Aβ reacted with BAN50 (Fig. 4), excluding the possibility that the Aβ is p3 (a major amino-terminally truncated form of Aβ), since BAN50 was raised against Aβ1–16. The Aβ also reacted with BC05 and appeared as a smear, as it did with BA27, although the density of the band was weaker than that observed upon reaction with BA27 (Fig. 4). These results indicate that the species of Aβ imunoprecipitated with 4G8 in the apical compartment of ΔC MDCK cell cultures are likely to be Aβ1–40 and Aβ1–42.

3.5. Effect of formic acid treatment on the BAN50 immunoprecipitation of Aβ secreted into the apical compartment of ΔC MDCK cell cultures

Based on the results shown in Fig. 2, we speculated that the Aβ secreted into the apical compartment of the ΔC MDCK cell cultures was not recognized by BAN50 due to its altered immunoreactivity although this Aβ has the full amino acid sequence of Aβ1–40. To test this possibility, we treated the 4G8 immunoprecipitates from the media of the apical and basolateral compartments of ΔC MDCK cell cultures with concentrated formic acid to restore normal Aβ immunoreactivity from the one which the peptide had adopted. Immunoprecipitation with BAN50 subsequent to this treatment resulted in a substantial increase in Aβ immunoreactivity on the Western blotting of the proteins from the medium of the apical compartment of ΔC MDCK cell cultures. The intensity of the immunoreactivity of the apical Aβ was comparable to that of the basolateral Aβ (Fig. 5).

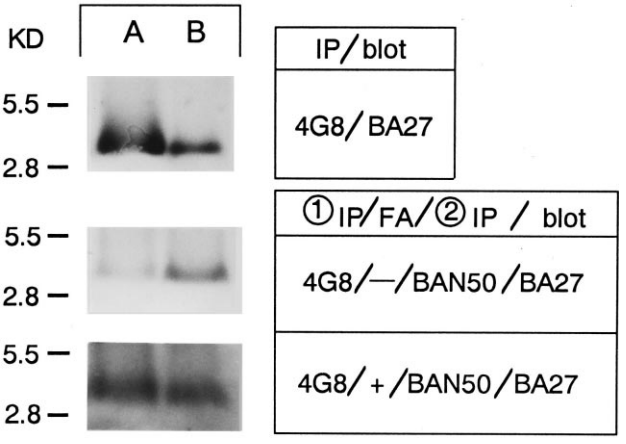

Fig. 5. Effect of formic acid treatment on Aβ immunoprecipitation with BAN50. The amount of Aβ which was reimmunoprecipitated with BAN50 in the apical compartment following 4G8 immunoprecipitation was increased to a level comparable to that in the basolateral compartment following formic acid treatment. (1)IP, first immunoprecipitation; (2)IP, reimmunoprecipitation of the first immunoprecipitates following solubilization in Laemmli sample buffer; FA, formic acid treatment.

3.6. Effect of inhibition of de novo cholesterol synthesis on the secretion of the Aβ from the apical surface of ΔC MDCK cells

Regarding the molecular mechanism of the generation of this unique Aβ species, we considered the possibility that the Aβ was generated via interaction with cholesterol and/or glycosphingolipids since these lipids are abundant in apical surface membrane and apically transported vesicles [10]. To examine this possibility, we first inhibited cholesterol synthesis using compactin. The optimum concentration of compactin for use in our experiments was determined as follows. The MDCK cells were treated with compactin at concentrations in a range from 100 nM to 5000 nM. The de novo cholesterol synthesis was decreased to approx. 40% of the control level by compactin at concentrations ranging from 500 nM to 1000 nM (Fig. 6a). The treatment of the cells with compactin at a concentration of 5000 nM caused marked cell death (data not shown). To investigate the role of cholesterol in the generation of this unique Aβ, ΔC MDCK cells were treated with compactin at concentrations of 500 nM and 1000 nM for 3 days. Incubation of the cells with compactin at the concentrations indicated above did not affect the cell viability, which was assayed using the calsein AM/ethidium homodimer staining kit (Molecular Probes, OR, USA) (data not shown). To exclude the possibility that treatment with compactin causes a change in the amount of Aβ secreted, the levels of secreted Aβ to the apical and basolateral compartments were deter-

mined using EIA. As shown in Fig. 6b, the amount of Aβ secreted to the apical and basolateral compartments was not altered following the treatment with compactin. However, interestingly, the Aβ immunoprecipitated with 4G8 from the medium of the apical compartment of ΔC MDCK cells which were treated with compactin was not detected as a smear on the Western blots (Fig. 6c). This effect of compactin on the disappearance of a smear on the blot was dose dependent. Furthermore, treatment of the cells with compactin increased the amount of Aβ species (Aβ40 and Aβ42) which were immunoprecipitated with BAN50 in the apical compartment of ΔC MDCK cell cultures (Fig. 7). The increase in the densities of the bands corresponding to Aβ were statistically significant (Aβ40: $P<0.01$; Aβ42: $P<0.01$).

3.7. *Effect of cholesterol binding on the secretion of the Aβ from the apical surface of ΔC MDCK cells*

To further confirm the involvement of cholesterol in the generation of the unique Aβ, ΔC MDCK cells were treated with filipin, a cholesterol-binding drug, at concentrations of 0.1 μg/ml and 0.3 μg/ml, for 3 days. As shown in Fig. 8, the Aβ secreted from the apical surface of ΔC MDCK cells ceased to appear as a smear on the blot in a dose-dependent manner. In contrast, the treatment with filipin did not cause any alterations in the appearance of Aβ secreted from basolateral surface (Fig. 8).

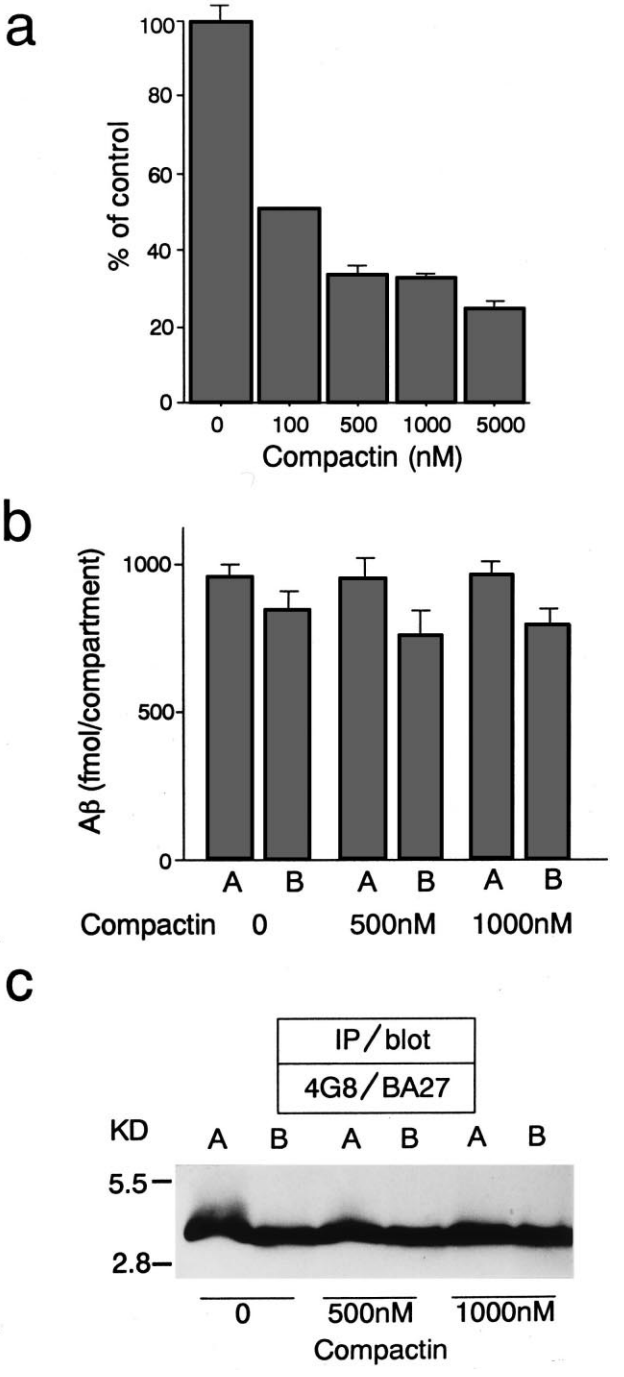

Fig. 6. Effects of inhibition of de novo cholesterol synthesis on the secretion of the Aβ from the apical surface of ΔC MDCK cells. (a) Inhibition of de novo cholesterol synthesis by compactin. The MDCK cells were cultured in media containing compactin at concentrations ranging from 100 nM to 5000 nM for 2 h at 37°C, and then exposed to 2 μCi of [^{14}C]acetate for a further 2 h. The incorporation of [^{14}C]acetate into newly synthesized cholesterol was determined by quantitating the radioactivity on thin layer chromatography plates using a Bio-imaging Analyzer System-2500 Mac. (b) Polarized secretion of Aβ40 from ΔC MDCK cells treated with compactin at the concentrations indicated. The two-site EIA was performed as described in Section 2. 4G8-coated multiwell plates were used. Note that the treatment of cells with compactin did not cause an alteration in the amount of Aβ40 secreted. Each column indicates the average of five values. (c) Effect of compactin on the appearance of the Aβ on the blots. The MDCK cells were cultured in the media containing compactin at concentrations of 500 nM or 1000 nM for 3 days. The Aβ in the apical (A) and basolateral (B) compartments were immunoprecipitated with 4G8. The blots of the immunoprecipitates were reacted with BA27. Note that the disappearance of the smear on the blots of Aβ from the apical compartment occurred in a dose-dependent manner, whereas there was no change in the appearance on the blots of Aβ from the basolateral compartment.

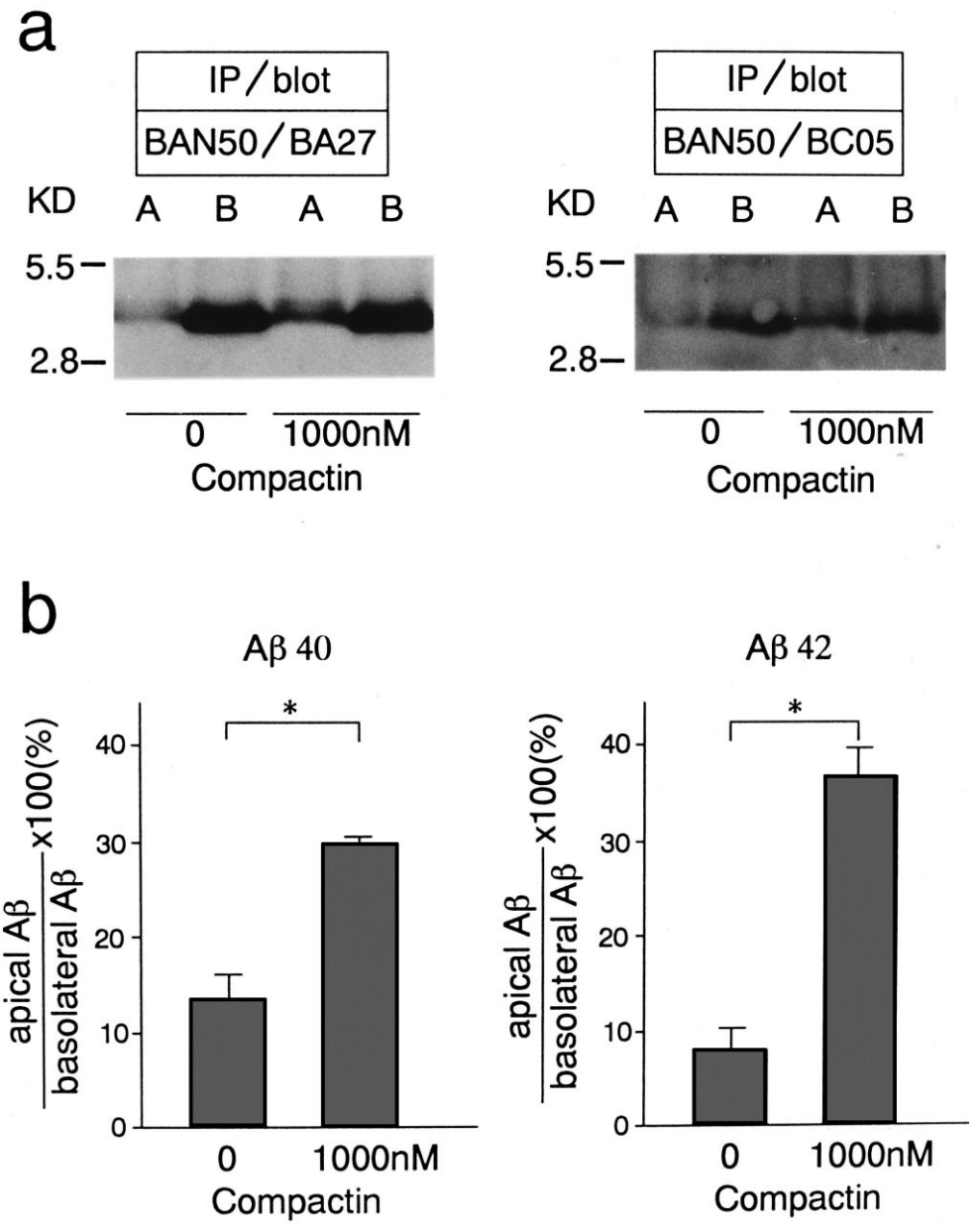

Fig. 7. Effect of inhibition of de novo cholesterol synthesis on the immunoprecipitation with BAN50. (a) The Aβ in the media was immunoprecipitated with BAN50, and the blot reacted with BA27 and BC05. Note that the intensities of both Aβ40 and Aβ42 in the apical compartment were increased by the treatment of cells with compactin, whereas there was no remarkable change in the intensities of the Aβs in the basolateral compartment. (b) The increase of the densities of the bands corresponding to the Aβs were statistically analyzed. The relative intensities of the Aβ (Aβ40 and Aβ42) detected in the medium of the apical compartment compared to those in the medium of the basolateral compartment were significantly increased upon treatment with compactin. Each column represents the average value obtained from five independent experiments. $*P < 0.01$(Aβ40); $*P < 0.01$(Aβ42).

4. Discussion

We are still far from achieving complete understanding of the molecular mechanism of physiological and pathological generation of Aβ. In this regard, we, here, present the following hypothetical model based on the results of this study (Fig. 9). A scenario is thus envisaged wherein Aβ is generated at two distinct sites, one before βAPP is sorted to the cell surface and the other on the cell surface through

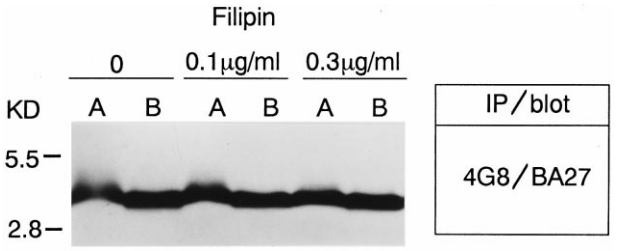

Fig. 8. Effect of cholesterol binding on the appearance of the Aβ secreted from ΔC MDCK cells on the blots. The ΔC MDCK cells were cultured in media containing filipin at concentrations of 0.1 μg/ml and 0.3 μg/ml for 3 days. The Aβ in the media was immunoprecipitated with 4G8 and the blot of the immunoprecipitates reacted with BA27. Note that the disappearance of the smear on the blots of Aβ from the apical compartment (A) occurred in a dose-dependent manner, whereas there was no change in the appearance of Aβ in the basolateral compartment (B).

internalization of βAPP. We are also the first, to our knowledge, to suggest the possibility that missorted βAPP is processed to generate an Aβ with unique molecular characteristics.

At this point, it is extremely difficult to elucidate the molecular mechanism of generation of this unique Aβ; however, it may be possible to assume the following. In ΔC MDCK cells, βAPP molecules may be inserted into apical transport vesicles [20], unique lipid microdomains called *rafts* (for review see [10]), which share the characteristic of a high cholesterol and glycosphingolipid content with caveolae or CLDs, and processed to generate the unique Aβ. This hypothesis is supported by a previous finding that some βAPP molecules in neurons are transported through axonal CLDs [21], which are analogous to apical transport vesicles or rafts in epithelial cells, although a report disputing βAPP localization in CLDs has also been published [22]. In the case of the wild-type MDCK cells, small amounts of βAPP molecules may be inserted into the microdomains, but the generation of the unique Aβ is likely to be precluded through the internalization of βAPP from the cell surface. Since ΔC βAPP is not internalized due to the lack of a cytoplasmic domain [3,9], it might be correct to state that not only missorting of βAPP, but also impairment of its internalization are prerequisites for the generation of the unique Aβ. In ΔC MDCK cells, the Aβ generated from the mis-

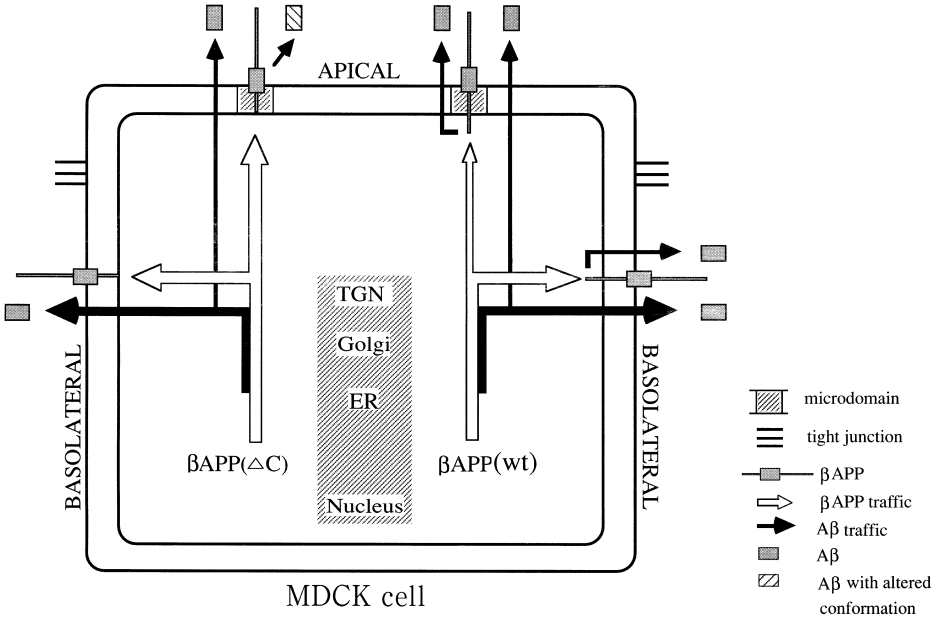

Fig. 9. A hypothetical model of the mechanism of Aβ generation. Aβ is generated at two distinct sites, one prior to and the other following sorting βAPP to the cell surface. In the latter case, the Aβ generation likely occurs through internalization of βAPP. The intracellular site of Aβ generation may be in the ER or the Golgi, since mutations of presenilin, which encodes a protein localized in the ER, cause abnormal generation of Aβ. In this study, conformationally altered Aβ was accumulated in the apical compartment whereas no such conformationally altered Aβ was detected in the basolateral chamber, indicating that an apical surface-destined microdomain comprised of lipid molecules including cholesterol and glycosphingolipids is a prerequisite for the generation of Aβ with an altered conformation.

sorted βAPP apparently acquires unique molecular characteristics including loss of BAN50 reactivity and appearance as a smear on immunoblotting, through interaction with some other elements localized in the lipid microdomains. The possibility of conversion of a constitutive protein to an abnormal, pathogenic form in such lipid microdomains is strongly supported by the report of the generation of PrP^{sc} from PrP^{c} in CLDs [11,12].

One of the most likely explanations for the loss of BAN50 reactivity is an association of the Aβ with other molecules, since this hypothesis would be consistent with the effect of formic acid treatment (Fig. 5), which strips off any binding protein or lipid molecules. We first considered the possibility of its association with GM1 ganglioside based on our previous findings [23,24]; however, we did not find any reactivity for GM1 ganglioside on the Aβ blots (data not shown). An association with other molecules is still possible. We may also have to pay attention to the possibility of the Aβ undergoing some undefined conformational transition since Aβ readily adopts altered secondary structures depending on the conditions of the environment [25–29] and association with lipid molecules [30]. If we hypothesize that the Aβ adopts an altered secondary structure, it would be of great interest to determine, in future studies, whether this novel Aβ is pathogenic since the amyloidogenic properties of Aβ are closely related to its secondary structure [31,32].

Some surprising results noted in this study are the effects of cholesterol depletion on the appearance of the unique molecular characteristics of the novel Aβ. Previous reports of caveolae disappearing in cholesterol-depleted cells [33], and of cholesterol depletion inhibiting the conversion of PrP^{c} to PrP^{sc} [11], taken together with the results of this study (Figs. 6–8), make it likely that the microdomains, with a high content of cholesterol, are the sites of generation of this unique Aβ. In regard to the pathological role of cholesterol in the development of AD, it is of particular interest that apolipoprotein E, the ε4 genotype of which is a major risk factor for the development of AD [34–36], is closely associated with cholesterol transport. In regard to the processing of βAPP, it was recently reported that this was modulated by a change in the cellular content of cholesterol [37,38]. Furthermore, alteration of cholesterol concentration in AD brains has also been reported previously [39]. Taken together with the results of this study, βAPP missorted to the cholesterol-rich cellular compartment may play a role in the pathogenesis of AD.

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