Deposition of aggregated amyloid β-protein (Aβ), a proteolytic cleavage product of the amyloid precursor protein (1), is a critical step in the development of Alzheimer’s disease (2). However, we are far from understanding the molecular mechanisms underlying the initiation of Aβ polymerization in vivo. Here, we report that a seeding Aβ, which catalyzes the fibrillogenesis of soluble Aβ, is generated from the apically missorted amyloid precursor protein in cultured epithelial cells. Furthermore, the generation of this Aβ depends exclusively on the presence of cholesterol in the cells. Taken together with mass spectrometric analysis of this novel Aβ and our recent study (3), it is suggested that a conformationally altered form of Aβ, which acts as a “seed” for amyloid fibril formation, is generated in intracellular cholesterol-rich microdomains.

Aβ is physiologically secreted into the extracellular space; however, why and how soluble Aβ aggregates and forms amyloid fibrils remains to be elucidated. A great deal of effort has been made to clarify this issue, using mainly in vitro systems. In most such experiments, it has been found that Aβ at much higher concentrations than those prevailing in biological fluids is needed for Aβ aggregation. Thus, it has been hypothesized that aggregation of soluble Aβ involves seeded polymerization (4, 5), although this assumption has not yet been proved in vivo.

We have recently reported the detection of a novel Aβ in the apical compartment of cultures of MDCK cells that had been stably transfected with APP cDNA (ΔC MDCK cell) with a truncated cytoplasmic domain (ΔC APP) (3). This Aβ species possesses unique molecular characteristics including its appearance as a smear on immunoblots and altered immunoreactivity. Significantly, these molecular characteristics disappeared dramatically following treatment of the cells with compactin or filipin, an inhibitor of de novo cholesterol synthesis and a cholesterol-binding drug, respectively. Based on previously reported evidence for ΔC APP being missorted to the apical surface (6) and the cholesterol concentrations of the apical plasma membrane and apical transport vesicles being higher than those in other cellular membranes (7), we concluded that a novel Aβ is generated from apically missorted APP in a cholesterol-dependent manner.

Regarding the involvement of cellular cholesterol in the generation of the pathogenic protein, it must be noted that cholesterol-rich lipid microdomains within cells, called caveolae-like domains, have been reported to be the likely sites of the conversion of the normal cellular form of prion protein (PrPC) to its pathogenic form (PrPSc) (8–11). Thus, it would be of great interest to investigate whether the novel Aβ detected in our recent study (3), the generation of which is exclusively dependent on the presence of cholesterol in the cell, has the potential to act as a seed for fibrillogenesis of soluble Aβ.

**EXPERIMENTAL PROCEDURES**

**MDCK Cell Culture—**Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum was used as the culture medium. We plated 2.5–3.0 × 10^5 MDCK cells transfected with human APP695 cDNA, with full-length APP (wild-type MDCK cell) or APP with a truncated cytoplasmic domain (ΔC MDCK cell) (12) onto 24-mm Transwell filters (Costar) and 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 electric resistance between the apical and basolateral compartments of the MDCK cell culture was performed by immersing electrodes into each of the compartments. The MDCK cells culture media were changed 3 days after plating, and the cells were cultured 24 h longer.

**Thioflavin T Assay and Congo Red Assay of Fibril Formation of Synthetic Aβ—**Thioflavin T assay was performed as described elsewhere (13), on a spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan). Optimum fluorescence measurements of amyloid fibrils were obtained at the excitation and emission wavelength of 446 nm and 490 nm, respectively, with the reaction mixture (1.0 ml) containing 1 μM thioflavin T (Nakalai tesque, Inc., Kyoto, Japan) and 50 mM of glycine-NaOH buffer, pH 8.5. Fluorescence was measured immediately after making the mixture and averaged for an initial 5 s. Synthetic Aβ (Aβ1–40, Bachem Switzerland) was initially dissolved in ice-cold distilled water at a concentration of 100, 200, and 300 μM, and then diluted with 9 volumes of PBS. Aliquots of the Aβ solutions were incubated in Eppendorf tubes at 37 °C. Every hour after the start of incubation, 10 μl of the solution of synthetic Aβ was taken and mixed with 990 μl of reaction mixture. The lot number of the Aβ used in the experiment of Fig. 1 was 518765 and that of the Aβ used in the other experiments was 510313. Peak fluorescence was dependent on the concentration of Aβ. We used 20 μM Aβ peptide for this study. Congo red assay was performed as described elsewhere (14).

**This paper is available on line at http://www.jbc.org**
formed essentially as described previously (3, 15). Briefly, 100 μl of the media of the MDCK cell cultures were diluted with 400 μl of buffer C (20 mM phosphate buffer (pH 7.0), 0.4 mM NaCl, 2 mM EDTA, 10% Block Ace (Dai-nippon, Tokyo, Japan), 0.2% bovine serum albumin, and 0.05% NaN₃), and 100 μl of the mixture were subjected to the multwell plates coated with either a monoclonal antibody specific for Aβ1–24 (3B) or anti-human IgG (Dai-nippon, Tokyo, Japan), 0.2% bovine serum for 2 h. Extraction of synthetic Aβ40 (Aβ1–40, Bachem Switzerland) was applied to the multwell plates for the construction of a standard curve. The plates were incubated at 4 °C overnight. After rinsing with PBS, loaded wells were reacted with appropriately diluted horseradish peroxidase-conjugated BA27, a monoclonal antibody specific for Aβ40, at 4 °C overnight. Bound enzyme activities were measured using the TMB Microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Effect of Addition of the Aβ Immunoprecipitated from the Medium of the MDCK Cells on the Fibril Formation of Synthetic Aβ—Aliquots (1.5 ml) of the medium from the transfected MDCK cell cultures were incubated with 4G8 (1.5 μg) at 4 °C overnight for Aβ immunoprecipitation 3 days after changing the medium. The mixtures were then incubated with protein G-Sepharose at 4 °C for 3 h and centrifuged. The pellets were washed thoroughly in RIPA buffer once, and then in Tris-saline buffer four times. The Aβ in the immunoprecipitates was extracted in 25 μl of a buffer containing 2% SDS by boiling. Following dilution with 975 μl of Tris-saline buffer, 5 μl of the solution (containing 1 pmol of the immunoprecipitated Aβ) was mixed with 10 μl of the synthetic Aβ solution (containing 2 nmol of Aβ1–40) and 85 μl of PBS buffer. The level of the immunoprecipitated Aβ was determined by enzyme immunosay (data not shown), and the molecular ratio between synthetic Aβ and the immunoprecipitated Aβ was approximately 2,000:1. Amyloid fibril formation of synthetic Aβ was quantitatively determined by measuring fluorescence intensity at 2 and 6 h after the incubation as described before. The background fluorescence intensity was determined using an extract of protein G-Sepharose that was incubated with 4G8 in fresh medium.

Inhibition of de Novo Cholesterol Synthesis—To inhibit de novo cholesterol synthesis, MDCK cells were incubated for 90 min with compactin (Sigma), a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, at concentrations of 0.5 and 1.0 μM, before changing the media to fresh ones. The cells were further cultured for 3 days with compactin, and then 1.5-ml aliquots of the medium from the apical and basolateral compartments of the culture were used for immunoprecipitation.

Treatment with Filipin—MDCK cells were cultured in medium containing filipin (Sigma), a polyene antibiotic that specifically binds to sterol, at concentrations of 0.1 and 0.3 μg/ml for 90 min before changing the media to fresh ones. The cells were further cultured for 3 days with filipin, and then 1.5-ml aliquots of the medium from the apical and basolateral compartments of the culture were used for immunoprecipitation.

Addition of Esogenous Cholesterol—To confirm that the seeding ability of the apical Aβ depends on the presence of cholesterol, we investigated the effect of compactin and filipin was reversed by the addition of exogenous free cholesterol.

Determination of the Total Cellular Level of Cholesterol and the Level of de Novo Cholesterol Synthesis—To determine the total level of cellular cholesterol in MDCK cells, cultures treated with compactin (1 μM) or filipin (0.3 μg/ml) for 2 days were washed 3 times in PBS and dried at room temperature. The samples were extracted with hexane/isopropanol (3:2 v/v) and dried with nitrogen. The total cholesterol levels in the samples were determined using a cholesterol determination kit (Kyowa Medical Co. Ltd., Tokyo, Japan). Protein concentration was determined using the BCA protein assay kit (Pierce), with bovine serum albumin as the standard. To determine the level of de novo cholesterol synthesis, MDCK cells were pretreated with compactin (1 μM) and filipin (0.3 μg/ml) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 2 h. 2 μCi/ml [3H]acetate was added into the cultures treated with the compounds. After 3 h of incubation, the cultures were washed three times in PBS and dried at room temperature. The samples were then extracted with hexane/isopropanol (3:2 v/v) and dried with nitrogen. The samples were quantitatively spotted on thin-layer chromatography plates and developed in a solvent system of hexane/ethanol/2-propanol/water (80:30:1). The radioactivities of the spots were detected and quantified by the Bio-imaging Analyzer System-2500 Mac (Fuji Film Co. Ltd., Tokyo, Japan).

Mass Spectrometry—Mass spectrometric analysis was performed essentially as described elsewhere (17). Aliquots of medium (5 ml) from the apical and basolateral compartments were incubated with 4G8 (5 μg) at 4 °C overnight for immunoprecipitation of Aβ. The mixtures were then incubated with protein G-Sepharose at 4 °C for 3 h and centrifuged. The pellets were washed thoroughly with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1.0% Nonidet P-40) once and then with distilled water four times. The immunoprecipitated Aβ was extracted with 10 μl of trifluoroacetic acid. 1 μl of the extracted solution was mixed with 10 μl of UV-laser desorption matrix (trifluoroacetic acid/water/acetonitrile 1:20:20, v/v/v), containing saturated a-cyano-4-hydroxycinnamic acid; 0.5 μl of this mixture was loaded onto the mass spectrometer sample probe and dried at room temperature. Mass spectra were measured using a UV-laser desorption/ionization time-of-flight mass spectrometer (Voyager Elite, PerSeptive Biosystem).

RESULTS AND DISCUSSION

MDCK cells were cultured as described previously (6). Stable transfection of these cells with APP cDNA was performed as described elsewhere (12). To investigate whether the novel Aβ in the medium of the apical compartment of the ΔC MDCK cell cultures, referred to as apical Aβ, potentially accelerates amyloid fibril formation of synthetic Aβ, we performed a thioflavin T assay as described previously (13), using the Aβ peptide immunoprecipitated from the conditioned media. As shown in Fig. 1a, when synthetic Aβ (Aβ1–40) was incubated with the
apical Aβ derived from the ΔC MDCK cells, the fluorescence increased without a lag phase and proceeded to equilibrium hyperbolically. This time-course curve suggests that the apical Aβ acts as a seed in this experiment. A perfect linear semilogarithmic plot ($r = 0.998$) shown in Fig. 1b, indicates that $F(t)$ satisfies a differential equation: $F^t(t) = B - CF(t)$, where $B$ and $C$ are constants (18). Based on this differential equation, amyloid fibril formation from synthetic Aβ incubated with the apical Aβ can be explained by a first-order kinetic model; i.e. the extension of amyloid fibrils may proceed via the consecutive association of synthetic Aβ first onto the apical Aβ then onto the ends of growing fibrils (18).

Electron microscopic analysis showed the formation of typical amyloid fibrils with a diameter of approximately 10 nm and helical structure (Fig. 1c, left panel). Similar helical filament structure was observed when 400 μM of synthetic Aβ (Aβ1–40) was incubated at pH 7.5, 37 °C for 3 days (18). When synthetic Aβ was incubated with other types of immunoprecipitated Aβ, the increase in the fluorescence was small and no fibrillar structures were observed with electron microscopic analysis (Fig. 1, a and c, right panel).

Accelerated fibrillogenesis upon addition of the apical Aβ was further confirmed using Congo red assay (data not shown). The extent of enhancement of fibrillogenesis by the apical Aβ was statistically significant (Fig. 2a). We excluded the possibility that these results were caused by alteration in the amount of Aβ secreted from the cells by determining the level of Aβ by enzyme immunoassay (Fig. 2b). To further confirm that the seeding ability of the apical Aβ depended on the presence of cholesterol, we performed an experiment to see if the effect of compactin and filipin was reversed by the addition of exogenous cholesterol. As shown in Fig. 3c, the inhibition of the apical Aβ-induced acceleration of fibrillogenesis of synthetic Aβ following treatment with compactin or filipin, was dramatically reduced by the addition of exogenous cholesterol. Total cellular level of cholesterol was not dramatically decreased in cultures treated with compactin or altered at all in those treated with filipin, whereas the de novo cholesterol synthesis was markedly suppressed in cultures treated with compactin (Fig. 3d). These results suggest that generation of the seeding Aβ requires the presence of cholesterol in specific microdomains and does not depend on the total cellular level of cholesterol. The altered Aβ species was also generated in cultures that were grown in media not

**Fig. 2. Statistical analysis of the acceleration of amyloid fibril formation by the apical Aβ.** Panel a, accelerated fibril formation from 20 μM of synthetic Aβ1–40 upon addition of Aβ immunoprecipitated from the ΔC MDCK cells. Each column represents the average ±1 S.E. of 4 values of the extent of increase in fluorescence over the background level obtained using protein G-Sepharose incubated in fresh media. Note that acceleration of the amyloid fibril formation was observed in the mixture containing Aβ immunoprecipitated from the medium of the apical compartment of the ΔC MDCK cells. *p < 0.01 (Student’s t test).

Panel b, determination of the level of Aβ40 secreted into the media of the MDCK cells by EIA. EIA was performed as described previously (15). Note that the levels of Aβ40 in the media were not correlated with the fluorescence intensities (panel a).

**Fig. 3. Cholesterol-dependent seeding ability of the apical Aβ.** Panel a, inhibition of the apical Aβ-induced acceleration of fibril formation of synthetic Aβ1–40 following treatment of the cells with compactin or filipin. Each column represents the average ±1 S.E. of 4 values of the fluorescence increase over the background level obtained using protein G-Sepharose incubated in fresh media. Panel b, determination of the level of Aβ40 secreted into the media of the MDCK cells by EIA. EIA was performed as described previously (15). Note that the levels of Aβ40 in the media were not correlated with the fluorescence intensities (panel a). Panel c, prevention of inhibition of the apical Aβ-induced acceleration of fibril formation by compactin and filipin, following the addition of exogenous cholesterol at the concentration indicated. Each column represents the average ±1 S.E. of 4 values as described in panel a. Panel d, determination of the total cellular level of cholesterol and the de novo cholesterol synthesis in cultures treated with compactin and filipin. Each column represents the average ±1 S.E. of 4 values. *p < 0.01; **p < 0.05 (Student’s t test).
Generation of a Seeding Amyloid β-Protein in Cell Culture

Here we report, for the first time, that a seeding Aβ depends exclusively on the presence of cholesterol as shown in Fig. 3, a and c. Determination of the intracellular site of the generation of the seeding Aβ remains to be determined; however, lipid microdomains, called rafts (20), sharing a high content of cholesterol and glycosphingolipid with caveola or caveola-like domains are likely to be the best candidate for the following reasons: first, the axonally sorted APP, analogous to apically sorted APP in epithelial cells, is conveyed via caveola-like domains in neurons (21); second, localization of APP in caveola has recently been reported (22); third, further evidence to support the generation of Aβ in the cholesterol-rich microdomains is accumulating (23, 24).

At this point, it is extremely difficult to elucidate the molecular mechanism of acquisition by the apical Aβ of its unique molecular characteristics, including its seeding ability; however, it may be reasonable to assume that the apical Aβ adopts an altered conformation based on the following experimental results obtained from this and previous studies (3): first, the immunoreactivity of the apical Aβ to BAN50, in addition to its smearing behavior on gel electrophoresis, changed following treatment of the cells with compactin without any alteration in its mass number (Ref. 3 and Fig. 4); second, the BAN50 immunoreactivity for the apical Aβ recovered following treatment of the Aβ with formic acid (3). In the putative conformational alteration of the apical Aβ, auxiliary factors localized in the lipid microdomains may be involved, as is suggested in the conversion of the normal cellular form of prion protein (PrP⁰) to its pathogenic form (PrPsc) (9). Among the candidates for such factors, we prefer to consider GM1 ganglioside for the following reasons: first, GM1 ganglioside is one of the main resident molecules in the microdomains (25); second, we have previously found GM1 ganglioside-bound Aβ in human brains in the early stages of Alzheimer’s disease (26); third, Aβ undergoes alteration of its secondary structure via interaction with GM1 ganglioside (27, 28); and fourth, it has recently been reported that amyloid fibril formation of Aβ is drastically accelerated in the presence of GM1 ganglioside (14). Thus, it is intriguing to speculate that the Aβ generated from the apically missorted APP undergoes conformational alteration via association with GM1 ganglioside in the lipid microdomains and then acts as a template for the consecutive conversion of a nascent soluble Aβ into a seeding Aβ.

Recently, much attention has been focused on the conformational alteration of constitutive proteins in the brain in various neurodegenerative diseases (4, 5, 29). In such processes, a constitutive protein in the brain undergoes minor perturbations of structure, leading to an increase in β-sheet content; it has been proposed that these disease processes be grouped into one new category, the conformational diseases (29). Conformational conversion of the prion protein with resultant aggregation of its pathogenic form is a well-known example belonging to this category (30). Although Alzheimer’s disease could also be included in the conformational diseases group (4, 5, 29), to date, no study has ever shown the generation of a conformationally altered isoform of Aβ with seeding ability. In this regard, our results present, for the first time, evidence for the generation of a seeding Aβ in cell culture, and furthermore, may be used to explain the molecular mechanism underlying initiation of amyloid fibril formation in vivo.

Finally, from the results of this and other (31) studies, one can consider the possibility that missorting of APP or altered intracellular trafficking of Aβ plays a role in the pathogenesis of Alzheimer’s disease. Further studies, using polarized differentiated neurons should be carried out to investigate the consequences of generation of a seeding Aβ from axonal or presynaptic membranes.
Acknowledgments—The authors thank Y. Hanai for preparing this manuscript and I. Yamaguchi for technical support in the electron microscopic analysis.

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