Long Amyloid β-Protein Secreted from Wild-Type Human Neuroblastoma IMR-32 Cells

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ABSTRACT: The 39- to 43-amino acid amyloid β-protein (Aβ) is deposited as amyloid in Alzheimer's disease. Recent studies have suggested that short Aβ ($Aβ_{39}$ or $Aβ_{40}$) and long Aβ ($Aβ_{42}$ or $Aβ_{43}$) play different roles in Alzheimer-type pathology. However, little attempt has been made to investigate the cellular mechanisms underlying the generation of short and long Aβ individually. In the present report, we first measured the amount of short and long Aβ that are secreted from wild-type human and rodent cells with neuron- or glia-like properties using highly sensitive sandwich-ELISAs that discriminate long Aβ from short Aβ. The results showed that long Aβ secreted by all cells constitutes approximately 10% of the total Aβ. To identify the molecular species of long Aβ, we next isolated the Aβ species secreted from human neuroblastoma IMR-32 cells by affinity chromatography, gel-filtration HPLC, and reverse-phase HPLC. Mass spectrometric analysis demonstrated unequivocally that IMR-32 cells produce $Aβ_{1-42}$ together with $Aβ_{1-37}$, $Aβ_{1-38}$, $Aβ_{1-39}$, and most predominantly, $Aβ_{1-40}$. Finally, to investigate the cellular mechanisms that generate $Aβ_{1-42}$, we studied the effects of brefeldin A and monensin on the production of $Aβ_{1-40}$ and $Aβ_{1-42}$ in IMR-32 cells. These reagents reduced the production of both $Aβ_{1-40}$ and $Aβ_{1-42}$ simultaneously in a concentration-dependent manner. These results indicate that processing of wild-type amyloid precursor protein normally generates $Aβ_{1-42}$ at a ratio of 10% to total Aβ and that $Aβ_{1-40}$ and $Aβ_{1-42}$ share a common secretory mechanism that involves acidic compartments such as the late Golgi or early endosomes.

The amyloid β-proteins (Aβs)[1] are 39- to 43-amino acid polypeptides deposited as aggregates in the brain of patients with Alzheimer's disease (AD). Many efforts to characterize the species of Aβ have shown that, in most instances, the primary component deposited as amyloid in cerebral vasculature is short Aβ, which possesses Val^{39} or Val^{40} as the C-terminus (for example, $Aβ_{1-39}$ or $Aβ_{1-40}$) (Prelli et al., 1988; Joachim et al., 1988; Miller et al., 1993). Recent investigations have revealed that Aβ also exists in a soluble form in biological fluids (Seubert et al., 1992), and the predominance of short Aβ in human cerebrospinal fluid was demonstrated by the mass spectrometric analysis (Vigo-Pelfrey et al., 1993). On the contrary, many lines of evidence have appeared to support the hypothesis that long Aβ, the C-terminus of which is a few residues longer than short Aβ (for example, $Aβ_{1-42}$ or $Aβ_{1-43}$), plays a key role in β-amyloidogenesis. Long Aβ has been found to be the species of Aβ that initially and predominantly accumulates in brain parenchyma (Iwatsubo et al., 1994, 1995; Glenner & Wong, 1984; Masters et al., 1985; Miller et al., 1993; Roher et al., 1993). Presence of long Aβ dramatically accelerates formation of Aβ fibrils by a nucleation-dependent mechanism (Jarrett et al., 1993). These results have suggested that short Aβ and long Aβ play different roles in Alzheimer-type pathology.

However, little attempt has been made to investigate the cellular mechanisms underlying the generation of short and long Aβ individually. Although secretion of Aβ by cultured cells has been elucidated previously (Haass et al., 1992), Aβ species generated by the cells remain largely uncharacterized. Dovey et al. (1993) have shown that human embryonic kidney cells overexpressing human amyloid precursor protein, APP_{751}, with the Swedish double mutation mainly produce $Aβ_{1-40}$ with a minor production of $Aβ_{1-42}$. However, it is reported that processing of APP by these peripheral cells differs from that of neuroglioma cells or human mixed brain cells (Knops et al., 1995). Therefore, to understand the pathologic roles of Aβ in AD brains, it is important to investigate Aβ species produced by wild-type cell lines with neuron- or glia-like properties. It seems also important to determine the ratios of long Aβ to total Aβ secreted by those cultured cells although the low levels of Aβ production in the cells have made it difficult for such studies.

To overcome this problem, we recently developed sandwich-ELISAs (Suzuki et al., 1994a,b; Iwatsubo et al., 1994) that specifically discriminate a low amount of soluble long Aβ from short Aβ. In the present study, we further developed sandwich-ELISAs that enable the detection of human and rodent Aβ. Using these ELISAs, we first measured levels of Aβ in conditioned media of several human and rodent cells with neuron- or glia-like properties. Long Aβ produced by wild-type human neuroblastoma IMR-32 cells was then isolated and further characterized using mass spectrometric

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[1] Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; APP, amyloid precursor protein; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FCS, fetal calf serum; GMBS, *N*-[(γ-maleimidobutyryl)oxy]succinimide; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

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analyses. The cellular mechanisms that generate the long Aβ were also investigated using several inhibitors for the trafficking of APP.

MATERIALS AND METHODS

Synthetic Peptides. For immunogens, $A\beta_{1-40}$, [Cys[17]]$A\beta_{1-16}$, and $A\beta_{25-35}$ were synthesized by an automated peptide synthesizer (Applied Biosystems Model 430A, Perkin Elmer Corp., Norwalk, CT). [Cys[34]]$A\beta_{35-43}$ was synthesized by using an *N*-[(9-fluorenylmethoxy)carbonyl (Fmoc)]-amino acid derivatives cartridge (Applied Biosystems). The peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ which are used as standards for sandwich-ELISAs were purchased from Bachem Feinchemikalien AG (Budendorf), and $A\beta_{1-28}$ was from Peninsula Laboratories (Belmont, CA). The synthesis of $A\beta_{1-43}$ was described elsewhere (Iwatsubo et al., 1994). $A\beta_{1-38}$ and $A\beta_{1-39}$ were prepared by limited proteolysis of $A\beta_{1-40}$ (Bachem) with carboxypeptidase Y, followed by purification with reverse-phase HPLC using a Vydac C4 column (The Separations Group, Hesperia, CA). [Cys[29]]$A\beta_{11-28}$ and $A\beta_{17-24}$ were purchased from Biologica (Nagoya), and [Cys[29]]$A\beta_{17-28}$ was from Accord (Tokyo).

Antibodies. All the anti-Aβ mouse monoclonal antibodies used in this report (excluding BAN052 and BNT77) have been described elsewhere (Suzuki et al., 1994a,b). For immunogens, [Cys[17]]$A\beta_{1-16}$ and [Cys[29]]$A\beta_{11-28}$ were conjugated with bovine thyroglobulin previously maleimidated with *N*-[(γ-maleimidobutyryl)oxy]succinimide (GMBS), and [Cys[34]]$A\beta_{35-43}$−bovine serum albumin (BSA) complex was prepared similarly. $A\beta_{1-40}$ and $A\beta_{25-35}$ were conjugated with bovine thyroglobulin with 0.3% glutaraldehyde as a coupling agent. These immunogens (80 μg/mouse), together with complete or incomplete Freund's adjuvant, were subcutaneously injected into BALB/c mice (female, 8 weeks old) at 3-week intervals. Four days after intravenously injecting each mouse with 200 μg of immunogen, spleen cells from each immunized mouse were fused with mouse myeloma cells P3-X63Ag8-U1, as described in the previous report (Suzuki et al., 1989). Monoclonal antibodies BAN50 (IgG1, κ), BC05 (IgG1, κ), BA27 (IgG2a, κ), BS85 (IgG1, κ), and BNT77 (IgA, κ) were selected from those directed against $A\beta_{1-16}$, $A\beta_{35-43}$, $A\beta_{1-40}$, $A\beta_{25-35}$, and $A\beta_{11-28}$, respectively. BAN052 (IgG1, κ) was also selected from those directed against $A\beta_{1-16}$. The antibodies were purified from ascites fluid with a Protein A-immobilized column (IPA-300, Repligen, Cambridge, MA).

Sandwich-ELISAs. Sandwich-ELISAs for $A\beta_{1-40}$ or $A\beta_{1-42(43)}$ have been described previously (Suzuki et al., 1994a,b; Iwatsubo et al., 1994). Briefly, the monoclonal antibody BAN50, directed against the N-terminal region of Aβ, was used for capture (first antibody). Antibodies BA27, BS85, and BC05, which were the monoclonal antibodies directed against the C-termini of Aβ, were labeled with horseradish peroxidase (HRP) (Boehringer Mannheim GmbH, Mannheim) and were used for detection (second antibody). Their conjugation with HRP was performed according to the method of Ichimori et al. (1987). Sandwich-ELISA systems are designated as first antibody/second antibody in the following text.

In sandwich-ELISAs, BAN50-coated 96-well microtest plates (A/S Nunc, Kamstrup) were prepared by incubation of 1.5 μg of BAN50 in 0.1 mL of 0.1 M sodium carbonate buffer (pH 9.6) in each well at 4 °C for 1 day. After washing the plate with phosphate-buffered saline (PBS) solution, 0.3 mL of a 1% (w/v) solution of Block Ace (Snow Brand Milk Products, Sapporo) in PBS containing 0.05% NaN_3 was added to each well, and the plate was stored at 4 °C until needed. To prepare standards, a 10 μM solution of $A\beta_{1-40}$ or $A\beta_{1-42}$ in dimethyl sulfoxide was stored at −80 °C and was diluted with buffer EC [0.02 M phosphate buffer (pH 7.0) containing 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace, 0.2% BSA, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.05% NaN_3]. Standard peptides or samples in 150 μL of buffer EC were put in each well of a BAN50-coated plate and incubated at 4 °C for 24 h. After being washed with PBS, the plate was reacted at 4 °C for 24 h with HRP-labeled BS85, BA27, or BC05 (100 μL in each well) diluted with buffer C [0.02 M phosphate buffer (pH 7.0) containing 0.4 M NaCl, 2 mM EDTA, and 1% BSA]. The plate was washed again with PBS, and bound enzyme activity was measured with a TMB microwell peroxidase system (Kirkegaard & Perry Lab, Gaithersburg, MD). The reaction of HRP was stopped with 1 M phosphoric acid (100 μL in each well), and absorbance at 450 nm was measured with a microplate reader (MTP-120, Corona, Tokyo). Another sandwich-ELISA system for measuring $A\beta_{40}$ or $A\beta_{42}$ that possesses a truncated or amino acid-substituted N-terminus was newly developed with BNT77 (anti-$A\beta_{11-28}$) instead of BAN50 in the ELISAs. Procedures used with this system were the same as described above.

Cell Culture. Human neuroblastoma IMR-32, SK-N-SH, SK-N-MC cells, murine neuroblastoma Neuro-2a, and NB41A3 cells, all from ATCC, were maintained at 37 °C, in an atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with nonessential amino acids, penicillin G (100 units/mL), streptomycin (100 μg/mL), and heat-inactivated fetal calf serum (FCS) (10%). Rat glioma C_6 cells from ATCC were maintained in Dulbecco's modified Eagle's medium which was supplemented with the antibiotics and heat-inactivated FCS as described above. Rat pheochromocytoma PC12h cells (Hatanaka, 1981) were kindly provided by Dr. Hatanaka. After cells became confluent in 25 cm^2 culture flasks, the culture fluids were replaced with fresh media and incubated for 48 h at 37 °C to condition the media for Aβ measurement.

In preparing IMR-32-conditioned medium to isolate Aβ, the culture medium was replaced with fresh medium containing 5% FCS after the cells became confluent. The conditioned medium was incubated for 48 h at 37 °C, harvested, and centrifuged (3000 *g*) for 30 min.

To study the effects of monensin, NH_4Cl, bafilomycin A_1, or brefeldin A on the production of Aβ by IMR-32 cells, the cells were cultured on a Linbro 24-well plate (ICN Biomedicals, Inc., CA). After the cells became confluent, the wells were washed with a serum-free medium, and 1 mL of the serum-free medium containing monensin, NH_4Cl, bafilomycin A_1, or brefeldin A of various concentrations was added. Each point was tested in triplicate. After 12 h incubation at 37 °C in 5% CO_2, the conditioned media were harvested, and the toxicity of these agents was then determined by the generation of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium (MTT) formazan (Mosmann, 1983). After centrifugation, aliquots of these conditioned media were

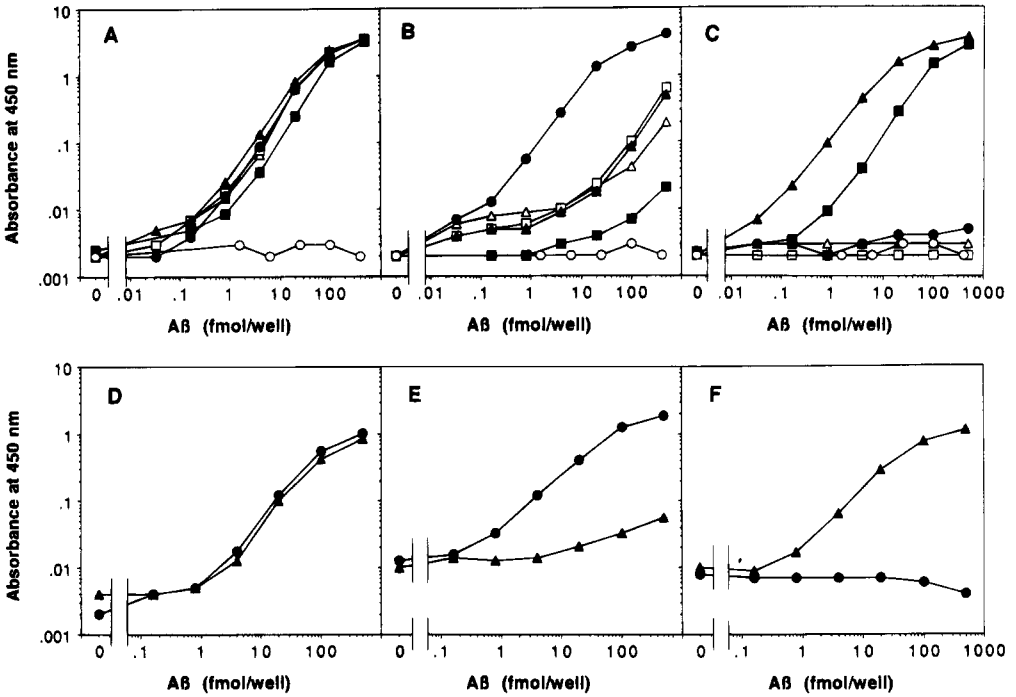

FIGURE 1: Standard curves of sandwich-ELISAs. Specificities and sensitivities for $A\beta_{1-28}$ (○), $A\beta_{1-38}$ (△), $A\beta_{1-39}$ (□), $A\beta_{1-40}$ (●), $A\beta_{1-42}$ (▲), and $A\beta_{1-43}$ (■) in sandwich-ELISAs of (A) BAN50/BS85 (1−16/25−35), (B) BAN50/BA27 (1−16/1−40), (C) BAN50/BC05 (1−16/35−43), (D) BNT77/BS85 (11−28/25−35), (E) BNT77/BA27 (11−28/1−40), and (F) BNT77/BC05 (11−28/35−43). Each antigen is shown in parentheses next to the antibody name.

subjected to BAN50/BA27 or BAN50/BC05 sandwich-ELISAs.

Isolation of Aβ from IMR-32-Conditioned Medium. Aβ in the IMR-32-conditioned media was affinity purified on a BAN052-immobilized column consisting of 1.4 mg of BAN052 and 160 μL of Tresyl Toyopearl resin (Toso, Tokyo). Adsorbed materials were eluted with 3 mL of a 60% solution of CH_3CN containing 0.2% trifluoroacetic acid (TFA), and the eluate was concentrated using a SpeedVac concentrator (SAVANT Instruments, NY). The concentrate was separated by gel-filtration HPLC on a TSK G3000PW column (7.5 × 300 mm; Toso) equilibrated with a 40% solution of CH_3CN containing 0.1% TFA. Aβ containing fractions identified by BAN50/BS85 ELISA were pooled and further fractionated by reverse-phase HPLC on a Vydac C4 column (4.6 × 250 mm). In the elution, the concentration of CH_3CN, containing 0.1% TFA, was kept at 16% for the first 10 min and then linearly increased from 16% to 20% for 5 min and from 20% to 35% during a 100-min period at a flow rate of 0.5 mL/min. Aliquots of fractions were used to analyze the concentration of Aβ using BAN50/BS85, BAN50/BA27, or BAN50/BC05 ELISA.

Mass Spectrometry. The mass of Aβ species produced by IMR-32 was determined by liquid secondary ion mass spectrometry using a double-focusing mass spectrometer (JEOL JMS-HX110HF, Tokyo) equipped with a cesium ion source. The instrument was operated at an accelerating voltage of 10 kV, at a mass resolution of 1:1000, and with 300 Hz filtering. The cesium gun was operated at 15 kV with a 2.2 A heater current. Aβ was isolated from 7.5 L of IMR-32-conditioned media as described above, and each of the immunopositive fractions was concentrated with a SpeedVac concentrator. After concentration, 1 μL of the sample solution was added to 1 μL of the liquid matrix (ratio 3:2, 3-nitrobenzyl alcohol/glycerol with 1% TFA) on a stainless steel probe tip. Mass spectra were obtained by scanning from *m/z* 4000 to 5000 at a rate of 10 s/scan, using a $(CsI)_n$ Cs^+ cluster ion for the calibration of mass number.

Amino Acid Sequencing. Automated Edman degradation was performed on a protein sequencer (Applied Biosystems 477A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems 120A).

RESULTS

Sandwich-ELISAs. We have established highly sensitive sandwich-ELISAs for Aβ using antibodies directed against $A\beta_{1-16}$ (BAN50), $A\beta_{25-35}$ (BS85), $A\beta_{1-40}$ (BA27), and $A\beta_{35-43}$ (BC05). Figure 1A−C show the standard curves of the sandwich-ELISAs using BAN50 as the first antibody. BAN50/BS85 ELISA detected both long and short Aβ beginning at Asp^1 in almost equal sensitivity (0.4 fmol/well). BAN50/BA27 ELISA detected $A\beta_{1-40}$ with much higher sensitivity (0.1 fmol/well), with a slight cross reactivity to $A\beta_{1-38}$, $A\beta_{1-39}$, $A\beta_{1-42}$, or $A\beta_{1-43}$. BAN50/BC05 ELISA detected $A\beta_{1-42}$ quite specifically and sensitively (0.1 fmol/well) without any cross reactivity to the short Aβs. $A\beta_{1-43}$ could be detected by BAN50/BC05 ELISA, but the precise reactivity has not as yet been determined because of the impurity of the standard peptide. These ELISAs were, however, specific for human Aβ species beginning at Asp^1. We next tried to develop sandwich-ELISAs that could detect not only $A\beta_{1-40}$ and $A\beta_{1-42}$ but also $A\beta_{40}$ or $A\beta_{42}$ with a truncated or modified N-terminus. For this purpose, we used BNT77 instead of BAN50, which was directed against $A\beta_{11-28}$ and did not react to $A\beta_{17-24}$ or $A\beta_{17-28}$, suggesting that its epitope is located in the N-terminal side of $A\beta_{11-28}$ (data not shown). Figures 1D−F show the standard curves of BNT77/BS85, BNT77/BA27, and BNT77/BC05 ELISAs for human $A\beta_{1-40}$ and $A\beta_{1-42}$. With respect to human Aβs beginning at Asp^1, the specificities of these ELISAs were

Table 1: Concentrations of Aβ Species in Culture Media Conditioned by Various Cell Lines

	BAN50			BNT77		
cell line	BC05 (fmol/mL)[a] $A\beta_{1-42}$	BA27 (fmol/mL)[a] $A\beta_{1-40}$	(%) $A\beta_{1-42}/(A\beta_{1-42} + A\beta_{1-40})$[b]	BC05 (fmol/mL)[a] $A\beta_{42}$	BA27 (fmol/mL)[a] $A\beta_{40}$	(%) $A\beta_{42}/(A\beta_{42} + A\beta_{40})$[c]
human						
IMR-32	19	140	11.9	39	270	12.6
SK-N-SH	5.2	57	8.3	<8	85	
SK-N-MC	<1.6	18		<8	48	
rat						
PC12h	<1.6	<1.6		30	291	9.3
C_6	<1.6	<1.6		17	170	9.1
mouse						
NB41A3	<1.6	<1.6		25	230	9.8
Neuro-2a	<1.6	<1.6		37	350	9.6

[a] Human synthetic Aβs were used as standards. [b] Calculated using the values from BAN50/BC05 and BAN50/BA27 ELISAs. [c] Calculated using the values from BNT77/BC05 and BNT77/BA27 ELISAs.

consistent with those using BAN50, whereas the sensitivities slightly decreased in the ELISAs using BNT77.

Production of Aβ Species by Various Cell Lines. We measured concentrations of short and long Aβs separately in the conditioned media of several human cell lines, including neuroblastoma IMR-32, SK-N-SH, and SK-N-MC cells, and in the media that were conditioned by rodent cell lines, including mouse neuroblastoma Neuro-2a, NB41A3, rat glioma C_6, and pheochromocytoma PC12h (Table 1). The human cell lines we studied secreted diverse amounts of Aβ. Among them, IMR-32 cells produced the largest amounts of short and long Aβ in both ELISA systems using BAN50 and BNT77 as the first antibody. In contrast to Aβ produced by human cells, rodent Aβ could be detected only by the ELISAs using BNT77 but not by those using BAN50. Considering the three amino acid substitutions of $Arg^5 \rightarrow Gly$, $Tyr^{10} \rightarrow Phe$, and $His^{13} \rightarrow Arg$ located in the N-terminal of rodent Aβ, it seems reasonable to suppose that BNT77/BA27 and BNT77/BC05 ELISAs can determine short and long Aβ independently of their N-terminal sequences. All the observations using BAN50 or BNT77 indicate that cultured cells with neuron- or glia-like properties secrete long Aβ as a minor species and that the percentage of long Aβ generated is constant at approximately 10% of total Aβ. Because IMR-32 cells secreted the largest amount of both Aβs compared with the other human cell lines, we chose them for further investigations of Aβ species.

Isolation of Soluble Aβ Secreted from IMR-32 Cells. One liter of medium conditioned by IMR-32 cells was prepared for the initial analysis of Aβs produced by IMR-32 cells. The Aβs in the medium were purified by affinity chromatography with a BAN052-immobilized column followed by gel-filtration HPLC on a TSK G3000PW column and by reverse-phase HPLC on a Vydac C4 column. Authentic $A\beta_{1-40}$ and $A\beta_{1-42}$ were also run on the same column. Figure 2 shows the elution profiles of immunoreactive Aβ secreted from IMR-32 cells separated on reverse-phase HPLC. Immunoreactivities detected by BAN50/BS85 ELISA agreed well with several peaks on the profile monitored at 210 nm. In BAN50/BA27 ELISA, a major peak appeared at the elution position of the authentic $A\beta_{1-40}$, which also corresponded to a major peak monitored at 210 nm. Similarly, BAN50/BC05 ELISA detected a major peak that eluted at the same elution time as the authentic $A\beta_{1-42}$. Broadening of immunoreactive peaks, however, may reflect

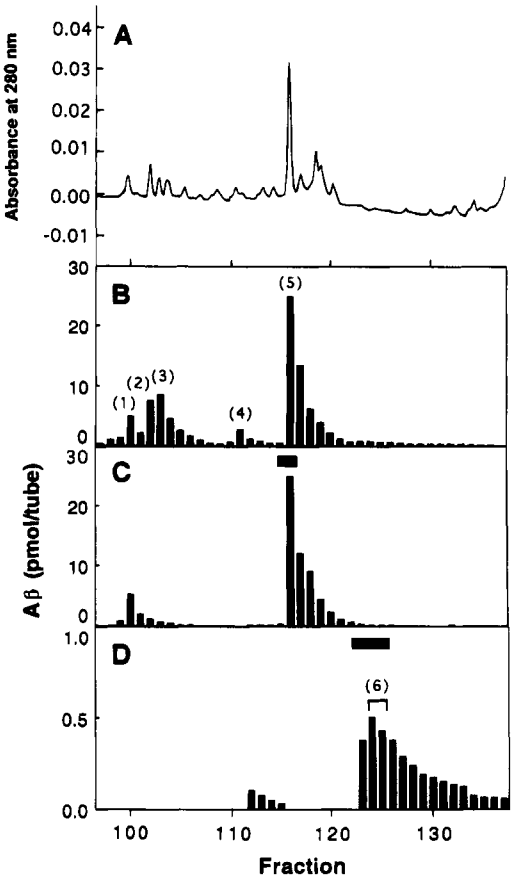

FIGURE 2: Reverse-phase HPLC analysis of immunoreactive Aβ secreted from IMR-32 cells. Aβs in the conditioned medium of IMR-32 cells were purified by affinity chromatography using a BAN052 column followed by gel-filtration HPLC using a TSK G3000PW column and by reverse-phase HPLC using a Vydac C4 column. Elution profiles of Aβs were monitored (A) at absorbance at 210 nm and determined by sandwich-ELISAs of (B) BAN50/BS85 (1−16/25−35), (C) BAN50/BA27 (1−16/1−40), and (D) BAN50/BC05 (1−16/35−43). Bars indicate elution positions of the authentic AB_{1-40} (C) and $A\beta_{1-42}$ (D).

small heterogeneity of the peptides. BAN50/BA27 ELISA detected a minor peak at fraction number 100, and BAN50/BC05 ELISAs detected a minor peak at fraction number 112, locations at which authentic $A\beta_{1-40}$ and $A\beta_{1-42}$ with oxidized Met^{35}, respectively, were eluted. These observations suggested that IMR-32 cells produce several Aβ species with different length in the C-terminal portion. It was obvious

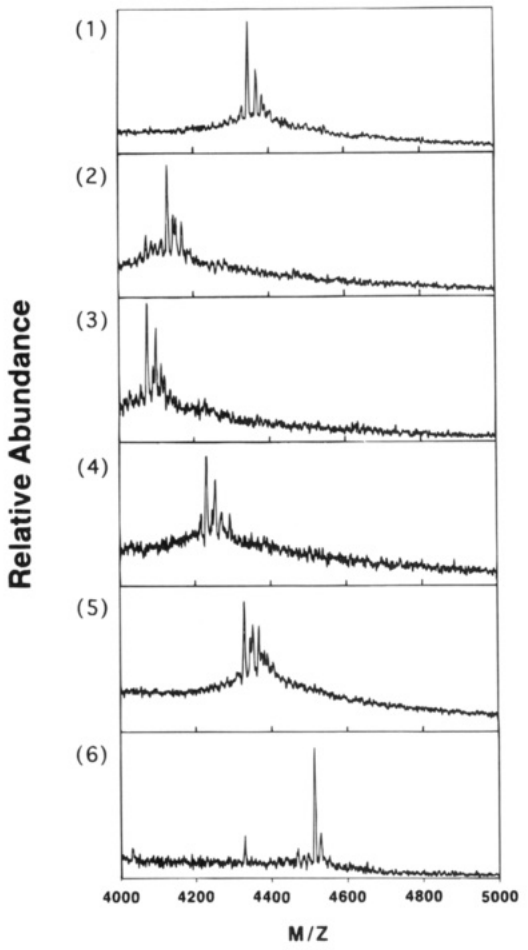

FIGURE 3: Mass spectrometric analyses of Aβ secreted from IMR-32 cells. Aliquots of the fractions of reverse-phase HPLC in Figure 2 were subjected to mass spectrometric analyses. The numbers from (1) to (6) correspond to the peak numbers shown in Figure 2. The spectrum consisted of several peaks, which were attributed to cationization ($[M + Na]^+$ and $[M + K]^+$) or Met^{35} oxidization or both, in addition to $[M + H]^+$. The immunoreactive Aβs contained in (1) Fr. 100, (2) Fr. 102, (3) Fr. 103, (4) Fr. 111, (5) Fr. 116, and (6) Fr. 124−125 were assigned to $Aβ_{1-40}$-oxide, $Aβ_{1-38}$, $Aβ_{1-37}$, $Aβ_{1-39}$, $Aβ_{1-40}$, and $Aβ_{1-42}$, respectively (see Table 2).

that long Aβ detected by BC05 was secreted from IMR-32 cells as a minor form together with the major BA27-immunoreactive species, supposedly $Aβ_{1-40}$.

We next tried to isolate Aβ from 7.5 Liters of IMR-32-conditioned medium to chemically characterize the species produced by IMR-32 cells. After performance of the same fractionating steps described above, each of the immunoreactive fractions detected by BAN50/BS85 ELISA in the reverse-phase HPLC was concentrated and analyzed by amino acid sequencing and by liquid secondary ion mass spectrometry. Amino acid sequencing revealed that Aβ species from these fractions possessed the N-terminal sequences of Aβ at its N-terminus. Figure 3 shows the results of mass spectrometric analysis. These spectra showed apparent signal complexities, which were attributed to cationization ($[M + Na]^+$ and $[M + K]^+$: where M represents the mass of the molecule) or oxidation of methionine or both, along with protonation. Therefore, it was concluded that each of the immunoreactive fractions contained single, major Aβ species. The $[M + H]^+$ values of the major peaks observed in the spectra provided good correspondence to the theoretical values of $Aβ_{1-40}$-oxide, $Aβ_{1-37}$, $Aβ_{1-38}$, $Aβ_{1-39}$, $Aβ_{1-40}$, and $Aβ_{1-42}$, with the

Table 2: Assignments of Aβ Species Secreted from IMR-32 Cells

HPLC fraction no.	obsd $(M + H)^+$ (*m/z*)	calcd $(M + H)^+$ (*m/z*)	assignments	immunoreactivity[a] BS85	BA27	BC05
(1) 100	4346.6	4346.9	$Aβ_{1-40}$-oxide	+	+	−
(2) 102	4132.5	4132.6	$Aβ_{1-38}$	+	±	−
(3) 103	4075.5	4075.6	$Aβ_{1-37}$	+	±	−
(4) 111	4232.1	4231.8	$Aβ_{1-39}$	+	±	−
(5) 116	4330.5	4330.9	$Aβ_{1-40}$	+	+	−
(6) 124−125	4514.2	4515.1	$Aβ_{1-42}$	+	±	+

[a] All Aβs in the HPLC fractions were detected by BS85 ELISA (+). Aβ immunoreactivity detected by BA27 and BC05 was classified into three groups according to the ratios (*R*) of immunoreactivity detected by BA27 vs BS85 and those detected by BC05 vs BS85. The symbols +, ±, and − were used as follows: +, $R > 0.15$; ±, $0.01 < R < 0.15$; −, $R < 0.01$.

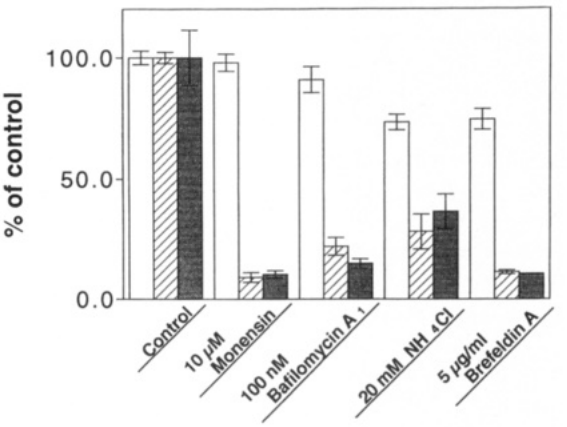

FIGURE 4: Decrease in secretion of long and short Aβ from IMR-32 cells cultured with APP processing inhibitors. The effects of 10 μM monensin, 100 nM bafilomycin A_1, 20 mM NH_4Cl, and 5 μg/mL brefeldin A on the secretion of short and long Aβ from IMR-32 cells were investigated. The cells were cultured for 12 h in the presence of the agents, and concentrations of short Aβ (hatched bars) and long Aβ (solid bars) in the conditioned media were measured by BAN50/BA27 and BAN50/BC05 ELISAs, respectively. After harvesting the conditioned medium, cytotoxicity of the agents was also measured by the MTT assays (open bars). Each point was tested in triplicate (mean ± SD).

differences between the observed and the theoretical values of $[M + H]^+$ measuring less than 0.9 atomic mass unit (Table 2). Namely, major Aβs that were immunoreactive to BA27 and BC05 were assigned to be $Aβ_{1-40}$ and $Aβ_{1-42}$, respectively; and Aβs that were immunoreactive to BS85 included $Aβ_{1-37}$, $Aβ_{1-38}$, and $Aβ_{1-39}$ in addition to $Aβ_{1-40}$ and $Aβ_{1-42}$. These results clearly indicate that IMR-32 cells produce $Aβ_{1-42}$ as a minor form together with a major form, $Aβ_{1-40}$.

Effects of Inhibitors for the Cellular Processing Pathway of APP on the Aβ Production of IMR-32 Cells. It is known that Aβ production is inhibited by monensin, NH_4Cl, or brefeldin A in several culture systems. To ascertain whether production of $Aβ_{1-42}$ is also affected by these agents, IMR-32 cells were cultured with the agents for 12 h, and $Aβ_{1-40}$ and $Aβ_{1-42}$ levels in the conditioned media were measured with the ELISAs. Although NH_4Cl and brefeldin A showed some cytotoxicity assayed with MTT, all of these agents reduced the levels of both $Aβ_{1-40}$ and $Aβ_{1-42}$ simultaneously (Figure 4). The effects of monensin and brefeldin A concentrations on the production of Aβ were investigated in detail (Figure 5). The levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ in the conditioned media were decreased in parallel: IC_{50} values associated with monensin were 0.05 μM for $Aβ_{1-40}$ and 0.04 μM for $Aβ_{1-42}$; IC_{50} values associated with brefeldin A were

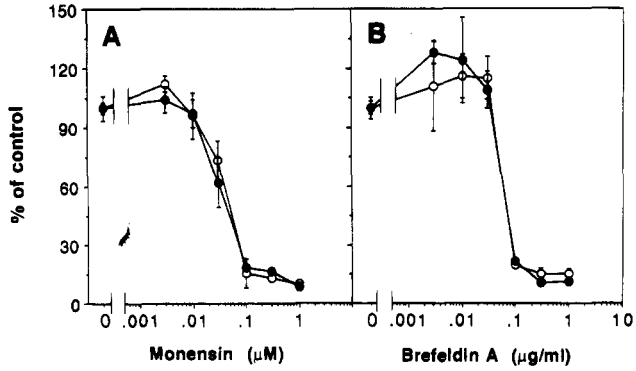

FIGURE 5: The effects of monensin and brefeldin A concentrations on APP processing to produce long and short Aβ. The effects of (A) monensin and (B) brefeldin A on the production of short (○) and long Aβ (●) were investigated by exposing the cells to the various concentrations of agents for 12 h. The long and short Aβ concentrations in the conditioned media were determined by BAN50/BA27 and BAN50/BC05 ELISAs, respectively. Each point was tested in triplicate (mean ± SD).

0.07 μg/mL for both $A\beta_{1-40}$ and $A\beta_{1-42}$. These results indicate that both $A\beta_{1-40}$ and $A\beta_{1-42}$ are produced through a similar processing of APP in acidic compartments.

DISCUSSION

It has become evident that long Aβ plays an important role in the pathogenesis of AD. In the present study, we showed that all the cells examined by us generated long Aβ at a ratio of approximately 10% of total Aβ, that long Aβ produced by IMR-32 cells was found to be $A\beta_{1-42}$, and that $A\beta_{1-40}$ and $A\beta_{1-42}$ shared a common secretory pathway that involved acidic compartments such as the late Golgi or early endosomes.

IMR-32 cells were reported to have neuron-like properties (Gotti et al., 1987) and to produce endogenously significant amounts of APP_{751} and APP_{695} (Shelton et al., 1990). Our experiments have demonstrated unequivocally that IMR-32 cells produce $A\beta_{1-42}$ together with $A\beta_{1-37}$, $A\beta_{1-38}$, $A\beta_{1-39}$, and most predominantly, $A\beta_{1-40}$. $A\beta_{1-43}$ was not found in the conditioned media of IMR-32 cells. The mass spectrometric assignment is highly accurate; the difference between the observed and calculated $[M + H]^+$ is less than 1 atomic mass unit. Similar results were reported using human embryonic kidney cells overexpressing APP_{751} with the Swedish double mutations, in which long Aβ produced by the cells was assigned as $A\beta_{1-42}$ by N-terminal amino acid sequences and by mass spectrometric analysis; the observed and calculated $[M + H]^+$ values for $A\beta_{1-42}$ were 4534 and 4516, respectively (Dovey et al., 1993). $A\beta_{1-43}$ was also not found in the conditioned media of the embryonic kidney cells. These results indicate that $A\beta_{42}$, a major species deposited in brains affected by AD, is secreted directly from the brain cells, and it is unlikely that $A\beta_{42}$ is generated from $A\beta_{43}$ or much longer species (for example, $A\beta_{44}$) by being trimmed by a carboxypeptidase at the C-terminus.

In addition to this C-terminal heterogeneity, it has been reported that the N-termini of Aβ in culture medium also vary considerably (Haass & Selkoe, 1993; Haass et al., 1994a, b; Busciglio et al., 1993). The relative abundance of the intact (starting at Asp^1), truncated (Phe^4, Arg^5, Glu^{11}), or extended (Val^{-3}, Ile^{-6}) Aβ was highly dependent on cell type. We measured the levels of Aβ in culture medium of IMR-32 cells using ELISAs consisting of BAN50 (anti-$A\beta_{1-16}$) and compared the results with levels determined by ELISAs using BNT77 (anti-$A\beta_{11-28}$). The latter ELISAs are expected to detect Aβs with the N-terminal heterogeneity. The BNT77 ELISAs showed levels of Aβ that were almost twice as high as the levels in BAN50 ELISAs, indicating that Aβ beginning at Asp^1 is one of the major species produced by IMR-32 cells. The remaining Aβ species detected by BNT77 should be investigated in a future study.

The ratio of $A\beta_{1-42}$ to the total amount of Aβ secreted by IMR-32 cells was found to be constant at approximately 10%. In all the human and rodent cell lines we investigated, the ratios of $A\beta_{1-42}$ or $A\beta_{42}$ to total Aβ were conserved at approximately 10%, although absolute values showed some diversity. Human teratocarcinoma NT2 cells differentiated with retinoic acid (NT2N) (Wertkin et al., 1993) also provided the same results (data not shown). Interestingly, the ratio of $A\beta_{1-42}$ to total Aβ in cerebrospinal fluid from individuals not affected by AD or in plasma of normal adults also appeared to remain constant at 10% (N. Suzuki, unpublished observations; M. Jensen, personal communications). These results indicate that the ratio of $A\beta_{1-42}$ to total Aβ is regulated to be 10% in vitro and also in vivo. In this regard, a major Aβ species, $A\beta_{1-40}$, might not play any important role in the deposition of Aβ in the aged brain and in AD because a primary and universal component in senile plaque is long Aβ (Iwatsubo et al., 1994, 1995; Gravina et al., 1995). In contrast, increased production of $A\beta_{1-42}$ might play a central role in Alzheimer-type disorders. This idea is supported by our recent finding that familial APP_{717} mutations consistently caused a 1.5- to 1.9-fold increase in the percentage of long Aβ generated (Suzuki et al., 1994a). The additional factors that promote the formation of fibrillar amyloid consisting of long Aβ, such as apoE4 and α1-antichymotrypsin, may be also responsible for the pathogenesis (Ma et al., 1994).

Currently, little is known about the cellular mechanisms for the production of Aβ (Haass & Selkoe, 1993). It has been reported that generation of Aβ is inhibited by brefeldin A (Haass et al., 1993), an inhibitor for trafficking of glycoproteins from the endoplasmic reticulum to the Golgi, and NH_4Cl (Haass et al., 1993) and monensin (Busciglio et al., 1993; Dyrks et al., 1993; Haass et al., 1993), which interfere with pH gradients in vesicular compartments. Therefore, the late Golgi or early endosomes are thought to be involved in the generation of Aβ (Haass & Selkoe, 1993; Haass et al., 1993). However, these studies failed to discriminate $A\beta_{1-42}$ from $A\beta_{1-40}$, indicating that their conclusions reflect the results of a major Aβ species such as $A\beta_{1-40}$. In this study, we investigated the effects of monensin, NH_4Cl, brefeldin A, and bafilomycin A_1 on the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ by IMR-32 cells. Bafilomycin A_1 is a specific inhibitor for vacuolar H^+-ATPase that also interferes with pH gradients in the central vacuolar system, including vesicular compartments (Bowman et al., 1988). The present investigation showed that the production of not only $A\beta_{1-40}$ but also $A\beta_{1-42}$ was inhibited by these agents. More importantly, through the actions of monensin and brefeldin A, $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were decreased in a parallel and a concentration-dependent manner. These results suggest that both $A\beta_{1-40}$ and $A\beta_{1-42}$ are produced under similar circumstances that are present in acidic compartments, such as the late Golgi or early endosomes.

The C-terminal cleavages that generate Aβ are thought to be mediated by an unidentified protease, designated γ-secretase. Our findings indicate that these proteases should produce Aβ_{1-42} at a ratio of 10% to total Aβ without generating Aβ_{1-43} under the acidic conditions. In this regard, we cannot provide support for the idea that cathepsin D is a candidate for γ-secretase, because the protease has been shown to favor generation of Aβ_{1-43} rather than Aβ_{1-42} (Ladror et al., 1994).

Recently, it has been reported that human APP with a Val^{717}→Phe mutation developed Alzheimer-type neuropathology in transgenic mice (Games et al., 1995). From our in vitro studies, the Val^{717}→Phe mutation has been found to produce the largest shift in the relative amount of long Aβ produced compared to the other APP$_{717}$ mutants (Suzuki et al., 1994b). If increased production of Aβ_{1-42} actually accelerates the course of Alzheimer-type disorders, it is important to identify extracellular and intracellular events that enhance the generation of Aβ_{1-42}. We found that several agents that inhibit processing of proteins in the acidic compartments did not change the ratio of Aβ_{1-42} to total Aβ in the medium. However, it is possible that the production of Aβ_{1-40} and Aβ_{1-42} is regulated differently under some circumstances. Further efforts should also be made to identify the γ-secretase responsible for generating Aβ_{1-40} or Aβ_{1-42} or both in Alzheimer-type disorders.

The sandwich-ELISAs that use BAN50 as the capture antibody are highly sensitive and specific for either Aβ_{1-40} or Aβ_{1-42}. Other sandwich-ELISAs using BNT77 instead of BAN50 are useful for the measurement of Aβ_{40} or Aβ_{42} that has a truncated N-terminus or an N-terminus with substituted amino acids, such as rodent Aβ. Our methods using the ELISAs and IMR-32 cells may provide an excellent system for identifying γ-secretase and for investigating factors that influence the production of Aβ_{1-42}. Our methods may also provide further information on the pathologic roles of Aβ_{1-42} in Alzheimer's disease.

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