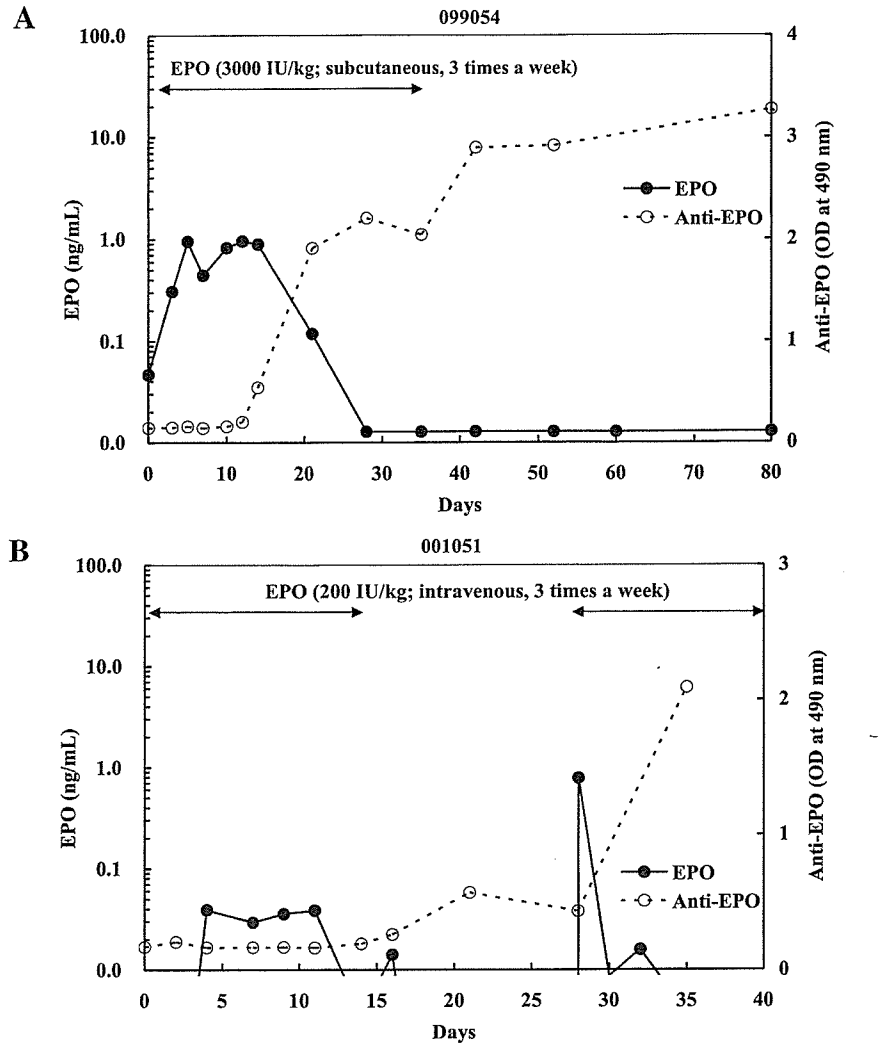

Fig. 1. Administration of only hEPO in cynomolgus monkeys. After subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero (A). Anti-hEPO antibody was also generated in another monkey (001051) receiving hEPO intravenously at a lower dose (200 IU/kg), leading to clearance of hEPO from the serum (B).

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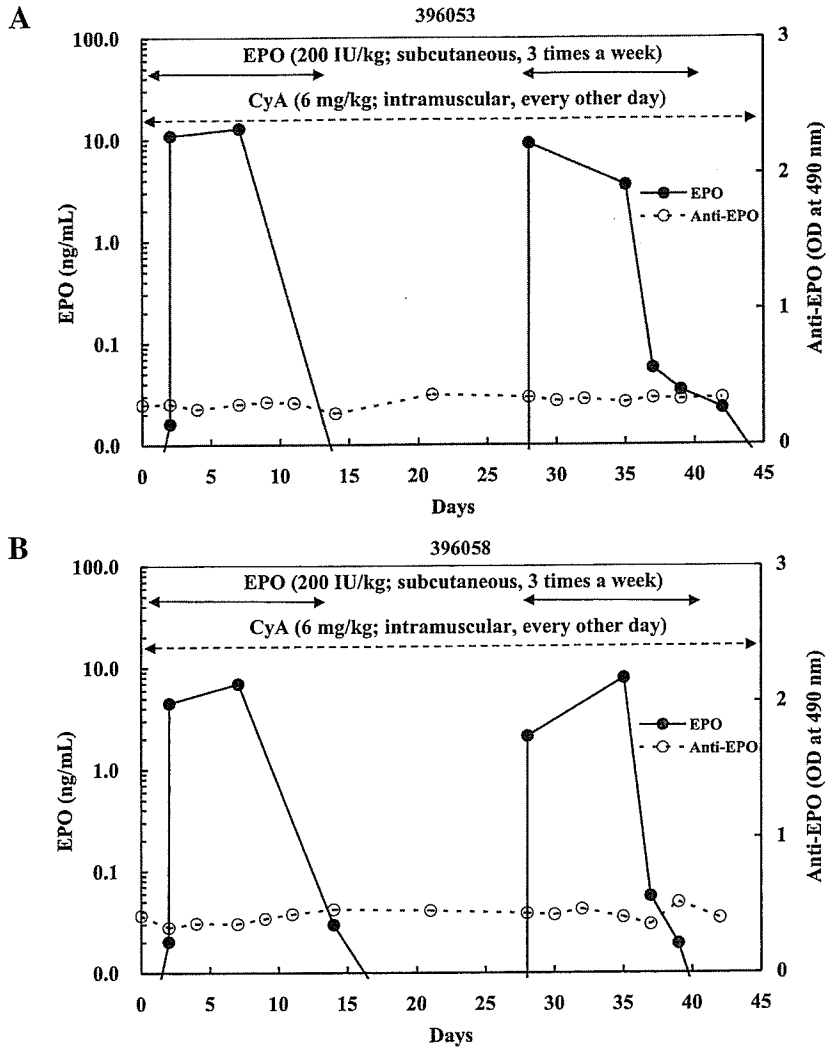

Fig. 2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by treatment with CyA in 2 cynomolgus monkeys (396053, 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of 200 to 400 *ng/ml*. Under the treatment with CyA, high serum levels of hEPO were obtained during hEPO administration. A second trial of hEPO administration resulted in a similar elevation of serum hEPO levels in 2 monkeys.

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Amyloid β Up-Regulates Brain-Derived Neurotrophic Factor Production From Astrocytes: Rescue From Amyloid β-Related Neuritic Degeneration

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Astrocytes, the most abundant type of glia in the brain, are considered to play a key role in Alzheimer's disease (AD) pathologies. In a cell culture study, we have previously shown that astroglial responses against amyloid β (Aβ) occur before obvious neuronal damage could be detected, suggesting the possibility that astrocytes might be an attractive therapeutic target for treating AD. In the present study, we investigated astroglial gene expression changes in response to Aβ to elucidate further the role of astrocytes in Aβ toxicity. By using real-time PCR and ELISA analyses, we found that Aβ rapidly induced astrocytes to produce brain-derived neurotrophic factor (BDNF). Aβ42 was more effective than Aβ40 in increasing astroglial BDNF production. Moreover, BDNF treatment rescued the neuronally differentiated human neuroblastoma cells from neuritic degeneration caused by Aβ toxicity. This is the first study to demonstrate that astrocytes are capable of increasing the production of a particular neurotrophic factor in response to Aβ. Our findings also identify BDNF as a potential therapeutic agent for preventing Aβ-related neuritic degeneration.

Key words: Alzheimer's disease; amyloid β; astrocytes; brain-derived neurotrophic factor; neuritic degeneration

Amyloid β peptide (Aβ) consists of 40–43 amino acids and is derived from amyloid precursor protein (APP; Citron et al., 1997). Aβ is the major protein component of senile plaques (SPs), a characteristic feature of Alzheimer's disease (AD; Glenner, 1988). Because Aβ is toxic to cultured nerve cells, some have argued that Aβ cytotoxicity is the major cause of brain damage observed in AD (Koh et al., 1990; Yankner et al., 1990; Behl et al., 1992; Mattson et al., 1992).

Astrocytes, the most abundant glial cell type in the brain, have various roles in maintaining normal brain physiology, such as forming growth tracts during development (Rakic, 1971, 1972; Silver and Sapiro, 1981; Hatten, 1985, 1990); forming the blood–brain barrier (Goldstein, 1987; Janzer and Raff, 1987); acting in immune responses like macrophages (Liu et al., 1989; Iacono et al., 1991; Lee et al., 1992); and producing neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF; Bruno et al., 2001; Mizuta et al., 2001; Matarredona et al., 2001). Many studies have shown that astrocytes are affected by soluble neuronal factors and several inflammation-associated cytokines (Eddleston and Mucke, 1993; Mark et al., 1995; McGeer and McGeer, 1995) and by Aβ (LaDu et al., 2001; Smits et al., 2002; Deb et al., 2003). Some of these studies also reported that astrocytes show immune responses against Aβ, an event that can be toxic to neighboring cells (LaDu et al., 2001; Smits et al., 2002; Deb et al., 2003). Other studies also demonstrated that astrocytes have an important role in clearing Aβ from the brain (Funato et al., 1998; Matsunaga et al., 2003; Wyss-Coray et al., 2003).

We have previously shown that astroglial responses against Aβ occur before obvious neuronal damage can be detected (Kimura et al., 2004). This finding suggests that the role of astrocytes during the early stages of AD pathology must be very important, implicating them as potential therapeutic targets for the treatment of AD. In the present study, we investigated how Aβ affects gene expression patterns in astrocytes during early stages of Aβ toxicity. We specifically focused our investigation on astrocyte-derived neurotrophic factors. From our screening, we

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identified a novel astroglial function in response to Aβ that has the potential for therapeutic applications.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under controlled conditions (24°C ± 1°C, 55% ± 5% humidity) in plastic cages with sterilized wood shavings for bedding. They were fed a commercially available diet (CMF; Oriental Yeast, Tokyo, Japan) and had ad libitum access to food and tap water. This study was conducted according to the guidelines of the Animal Care and Use Committee of the National Institute of Biomedical Innovation, Japan.

Rat Primary Cerebral Cortical Cultures and Astrocyte Cultures

On gestational day 18, pregnant rats were anesthetized with diethyl ether anesthesia, and fetuses were removed. The fetal brains were removed, then transferred into ice-cold isolation medium (IM) consisting of equal volumes of Ca^{2+}-free phosphate-buffered saline (PBS); Mg^{2+}-free PBS; and Dulbecco's modified Eagle's medium containing 1.2 mg/ml $NaHCO_3$, 110 μg/ml pyruvic acid, 25 μg/ml streptomycin, and 50 U/ml penicillin (mDMEM). After bisection of the brains into cerebral hemispheres, the meninges, hippocampi, and other subcortical structures were carefully removed, and the cerebral cortices were rinsed in culture medium (CM: mDMEM with 5% fetal calf serum) and minced into small pieces (<1 mm^3) in CM. The tissue pieces were digested at 32°C for 30 min in PBS containing 1.5 U/ml papain (Washington Biochemical Corporation, Lakewood, NJ), 0.1 mg/ml DNase I (Roche Diagnostics Japan), 0.2 mg/ml cysteine, 0.2 mg/ml albumin, and 5 mg/ml glucose. Cells were dissociated gently by passing the mixture several times through a disposable pipette, and then the mixture was centrifuged three times in CM at 800 rpm for 5 min at 32°C. For Western blot analyses, cells were plated at 4.2×10^5 cells/cm^2 onto culture dishes coated with 0.125% polyethylenimine. All cultures were maintained at 37°C in a humidified chamber containing 95% air and 5% CO_2. A half-volume of culture supernatant was replaced with prewarmed CM once per week.

These primary rat cerebral cortical cultures consisted mainly of neurons (>90%), with some astrocytes. We previously showed that these neuronal cells have complicated interactions with glia and other neurons and make synaptic connections with other neurons similar to those in vivo (Negishi et al., 2002).

To establish astrocyte cultures, we utilized the rat primary cerebral cortical cultures via the following procedure. After 14 days of culturing, cerebral cortical cells were dissociated with 0.025% trypsin (Invitrogen, Carlsbad, CA) and washed several times in CM. By these means, proliferating type 1 astrocytes were quickly selected from this suspension (Negishi et al., 2003). After maintaining them for a further 7 days, the cells were subcultured one more time for 7 days and plated at 4.2×10^5 cells/cm^2 in CM onto uncoated culture dishes. A half-volume of culture supernatant was replaced with prewarmed CM once per week.

Generation of Neuronally Differentiated SH-SY5Y Cells

SH-SY5Y neuroblastoma cells were purchased from American Type Culture Collection (ATCC, Vienna, VA). Cells were seeded at 1.0×10^4 cells/cm^2 onto culture dishes coated with collagen type I (Greiner Bio-One Germany) for Western blot analyses and also were plated at 5.0×10^4 cells/cm^2 onto Lab-Tek chamber slides (Nalge Nunc, Tokyo, Japan) coated with 0.5% polyethylenimine for immunocytochemical studies. After overnight incubation, cells were treated in CM containing 10 μM all-trans-retinoic acid (RA; Acros Organics) for 5 days. The cells were then washed and incubated with 50 ng/ml human brain-derived neurotrophic factor (BDNF; Sigma, St. Louis, MO) in serum-free CM containing 1% Insulin-Transferrin-Selenium-X Supplement (ITS-X; Invitrogen) for an additional 5 days. Then, medium was replaced with serum-free CM containing 1% ITS-X after a brief wash and maintained for another 1 day.

Aβ Treatment

Aβ peptides Aβ1–40 (Aβ40) and Aβ1–42 (Aβ42; Bachem, Torrance, CA) were dissolved in 100% dimethylsulfoxide (DMSO), then diluted in CM (final concentration: 0.45% DMSO). These Aβ40 and Aβ42 (not preaggregated) peptides were ultimately added to primary cortical cultures, astrocyte cultures, and neuronally differentiated SH-SY5Y cells. For molecular biological studies and Western blotting, CM containing Aβ peptides (5 μM) was added to primary cortical and astrocyte cultures, and these cultures were maintained for 3 and 24 hr. For BDNF-ELISA study, CM containing Aβ peptides (50 nM, 100 nM, 500 nM, 1 μM, and 5 μM) was added to astrocyte cultures, and these cultures were maintained for 3, 12, and 24 hr. For neuronally differentiated SH-SY5Y cells, serum-free CM containing Aβ42 peptide (10 μM) was added, and these cultures were maintained for 3 and 24 hr. The medium added to control cultures contained the same concentration of DMSO (0.45%) as cultures receiving the Aβ treatment.

Real-Time Quantitative Fluorescence-Based PCR

Total cellular RNAs from untreated and Aβ-treated astrocytes were isolated by using Trizol reagent (Invitrogen). The first-strand cDNA was synthesized from 3 μg total RNA with SuperScript II (Invitrogen). Expression levels of representative genes in rat astrocyte cultures were quantified with fluorescence-based real-time PCR with a Smart Cycler System (Applied Cepheid) and Sybr Premix ExTaq (Takara, Shiga, Japan). Primers designed in Oligo 6.0 primer analysis software (Molecular Biology Insights) for each gene analyzed were as follows: nerve growth factor-beta (NGFbeta) (+), 5′-CCAAGCACTGGAACTCATACTGC-3′; NGFbeta (–), 5′-CTGCTGAGCACACACACGCAG-3′; BDNF (+), 5′-GGAGGCTAAGTGGAGCTGACATAC-3′; BDNF (–), 5′-GTGCTTCCGAGCCTTCCTTTAGG-3′; glial cell line-derived CGGTTCCTGTG-3′; GDNF (–), 5′-CGACCGGCCTGCAACATGCCTG-3′; β-actin (+), 5′-GGAGTGGTTTGAG-

GTGTTGAGG-3′; β-actin (–), 5′-CCACACCCAGTAGAAGCCACAG-3′.

The shuttle PCR conditions were as follows: denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 sec and extension at 68°C for 15 sec. After shuttle PCR, a melting curve was constructed by increasing the temperature from 68°C to 95°C. PCR was repeated three to five times for each gene, and average copy numbers and standard deviations were calculated.

BDNF-ELISA

We used a sandwich enzyme-linked immunosorbent assay (ELISA) to assess BDNF production from astrocytes treated with Aβ. We used the Chemikine Brain-Derived Neurotrophic Factor Sandwich ELISA Kit (Chemicon, Temecula, CA) for our ELISA analyses according to the manufacturer's instructions. After 3, 12, or 24 hr of buffer or Aβ treatment, culture media from untreated and Aβ-treated astrocyte cultures were harvested and then used for the sandwich ELISA studies. BDNF levels were determined by referring to the standard curve. We performed three independent experiments (N = 6 for each experimental group), duplicating each experiment.

Neurotrophic Factor Treatment

To examine whether astrocyte-derived neurotrophic factors such as NGF, BDNF, and GDNF restore neuritic degeneration induced by Aβ treatment, we used the conditioned medium containing these factors to treat neuronally differentiated SH-SY5Y cultures. After 3 hr of Aβ treatment (10 μM), a half-volume of culture supernatant was replaced with prewarmed serum-free CM containing NGF, BDNF, or GDNF (final concentration of each was 50 ng/ml), and then cultures were maintained for another 21 hr (total culturing time, including Aβ treatment time, was 24 hr). The medium of the control cultures contained the same concentration of DMSO (0.45%) as cultures receiving Aβ treatment. This medium was replaced with prewarmed serum-free CM without neurotrophic factors. The medium of negative control cultures (i.e., cells receiving Aβ treatment alone) was also replaced with prewarmed serum-free CM without neurotrophic factors.

Western Blot Analyses

For Western blotting, the following antibodies were used: rabbit polyclonal antineurotrophic tyrosine kinase receptor, type2 (TrkB; Upstate, Lake Placid, NY); rabbit polyclonal anti-p75 neurotrophin receptor (p75NTR; Sigma); rabbit polyclonal anti-synapsin I (SynI; Chemicon); and mouse monoclonal anti-β-actin (AC15; Sigma). To extract total cellular proteins from the cultured cells, the cells were bathed in a solution containing 9.85 mg/ml Tris-HCl, 0.774 mg/ml ethylenediaminetetraacetic acid (EDTA), 0.348 mg/ml ammonium persulfate, 0.5% (v/v) Triton X-100, and 2.3% (w/v) sodium dodecyl sulfate (SDS) in PBS. Total proteins were isolated by centrifugation, adjusted to 10 μg, then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE with 10% acrylamide gel). Separated proteins were blotted onto polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween-20 overnight at 4°C, then incubated with primary antibodies (TrkB, 1:2,000; p75NTR, 1:1,000; SynI, 1:8,000; AC15, 1:20,000) for 1 hr at room temperature. They were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or mouse anti-rabbit IgG (1:6,000; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hr at room temperature. Immunoreactive elements were visualized by using enhanced chemiluminescence (ECL plus; Amersham). We performed two independent experiments (N = 6 for each experimental group), duplicating each experiment.

Immunocytochemistry

For immunocytochemistry, a mouse monoclonal antisynaptophysin antibody (SY38; Dako, Glostrup, Denmark) was used. Cells plated on chamber slides were fixed with methanol at −20°C, then incubated in primary antibody solution overnight at 4°C. Primary antibody dilution for SY38 was 1:500. After brief washes with buffer, sections were then sequentially incubated with Alexa 488-conjugated goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) and DAPI (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at room temperature. The sections were examined with a digital eclipse C1 confocal microscope (Nikon).

Data Analyses

For statistical analyses, one-way ANOVAs were performed, followed by the Bonferroni/Dunn post hoc test. Data are shown as means ± SD.

RESULTS

Aβ Induces BDNF Production From Astrocytes

Real-time PCR analyses of astrocyte cultures treated with Aβ for 3 hr indicated that Aβ induced BDNF gene expression (Fig. 1A). Aβ42, in particular, significantly increased BDNF expression in astrocytes (Fig. 1A). Neither Aβ42 nor Aβ40, however, affected NGF or GDNF gene expression (Fig. 1A). In contrast to cultures treated with Aβ for 3 hr, those treated for 24 hr did not display elevated neurotrophic factor expression (Fig. 1A). To confirm that Aβ increases the production and secretion of BDNF protein from astrocytes, we measured BDNF protein levels by using a sandwich ELISA system. BDNF-ELISAs indicated that Aβ treatment significantly increased BDNF production and secretion from astrocyte cultures after 3 hr, with Aβ42 inducing a larger increase in BDNF secretion than Aβ40 (Fig. 1B). However, after 12 hr and 24 hr, the BDNF secretion level dropped to almost the same level as control (data not shown). We further assessed the dose response of BDNF secretion against Aβ treatment after 3 hr (Fig. 1C). Even at 50 nM, Aβ42 was effective in increasing BDNF secretion from astrocyte cultures to the level observed at 5 μM (Fig. 1C). These results indicate that astrocytes respond fairly quickly (within 3 hr in our test system) to elevated levels of Aβ by up-regulating BDNF expression.

To determine whether the increased BDNF expression we observed in Aβ-treated astrocytes was mirrored by an increased expression of BDNF receptors, we assessed the

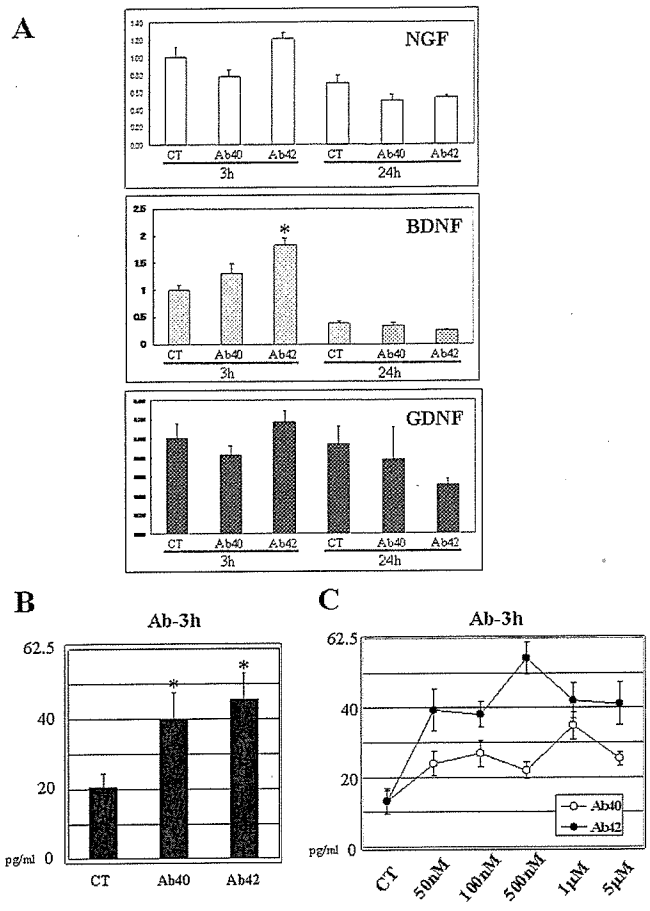

Fig. 1. **A:** Aβ induces BDNF expression and production in astrocytes. Expression patterns of NGF, BDNF, and GDNF were assessed with real-time PCR. Aβ42 treatment significantly increased BDNF gene expression in rat astrocyte cultures after 3 hr of treatment. Y-axes were normalized to each control value after 3 hr of treatment, and all data were also normalized according to β-actin mRNA levels (control group; CT). Values are means ± SD. $*P < 0.02$. **B:** Aβ also induced BDNF protein production and secretion in astrocytes. Protein expression was assessed with BDNF-ELISAs, and protein levels were calculated by using a standard curve. Both Aβ40 and Aβ42 significantly up-regulated BDNF production from rat astrocyte cultures after 3 hr of treatment. All data were normalized according to β-actin protein levels (control group; CT). Values are means ± SD. $*P < 0.02$. **C:** The dose-response curve relating Aβ concentration to BDNF production from rat astrocyte cultures after 3 hr of treatment. Aβ42 up-regulated BDNF production even at 50 nM as much as at 5 μM. All data were normalized according to β-actin protein levels (control group; CT). CT, controls consisted of extracts from cultures grown in standard culture medium with DMSO; Ab40, extracts from cultures treated with Aβ40; Ab42, extracts from cultures treated with Aβ42; Ab-3 hr, after 3 hr of Aβ treatment.

expression of two BDNF receptors, TrkB and p75NTR, in astrocytes and neurons. Western blot analyses showed that Aβ failed to induce TrkB expression in both astrocytes and neurons after 3 hr of treatment (Fig. 2). As expected, astrocyte and neuronal cultures treated with Aβ for 24 hr did not produce changes in TrkB expression (data not shown).

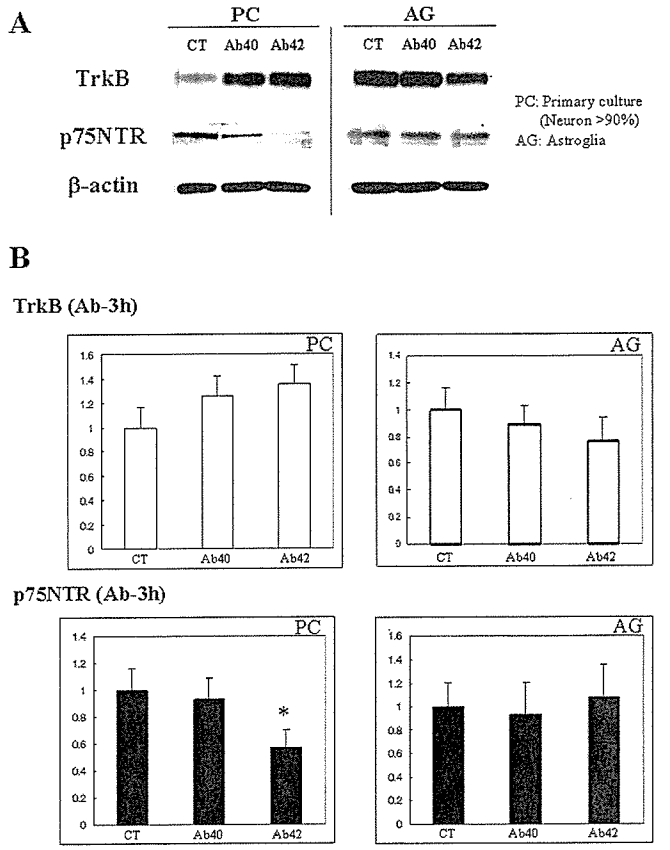

Fig. 2. **A:** Western blots showing the expression of TrkB, p75NTR, and β-actin in extracts from rat primary cerebral cortical (PC) cultures and astrocyte (AG) cultures following treatment with Aβ. In this experiment, Aβ40 and Aβ42 were diluted in culture medium to a final concentration of 5 μM. Although Aβ seemed to induce TrkB expression in cortical cultures after 3 hr of treatment, it failed to induce TrkB expression in astrocyte cultures. Aβ42 treatment reduced p75NTR expression in cortical cultures, but not in astrocyte cultures, after 3 hr of treatment. **B:** Histograms showing TrkB and p75NTR expression levels in PC and AG cultures after 3 hr of Aβ treatment. Data analyses indicated that the Aβ-induced increase in TrkB expression in cortical cultures was not significant. However, Aβ42 significantly reduced p75NTR expression. All data were normalized according to β-actin levels (control group; CT). Values are means ± SD. $*P < 0.02$. CT, control contained the same concentration of DMSO found in the Aβ treatments; Ab40, Aβ40 treatment; Ab42, Aβ42 treatment; Ab-3 hr, after 3 hr of Aβ treatment.

BDNF is the ligand not only for TrkB but also for p75NTR. Whereas BDNF binding to TrkB initiates neurotrophic signaling, BDNF binding to p75NTR initiates apoptotic signaling (Huang and Reichardt, 2003; Nykjaer et al., 2005; Woo et al., 2005). Unlike its effects on TrkB, Aβ42 significantly decreased p75NTR expression in cortical cultures, but not in astrocyte cultures, after 3 hr of treatment (Fig. 2). Taken together, our results suggest that astrocytes and neurons respond rapidly to Aβ42, within 3 hr of exposure—Aβ42 increased BDNF production in astrocytes, and decreased p75NTR expression in neurons.

BDNF Rescues Neuronally Differentiated Human Cells From Neuritic Degeneration Caused by Aβ

To test BDNF as a potential therapeutic agent for treating Aβ-related neuronal toxicity, we assessed the ability of BDNF to restore Aβ-related neuritic degeneration in neuronally differentiated human cells, SH-SY5Y cells. We treated our cultures with Aβ42, because Aβ42 is more closely associated with AD pathogenesis than is Aβ40 (Burdick et al., 1992; Jarrett et al., 1993; Suzuki et al., 1994; Younkin, 1994).

Western blot analyses confirmed that Aβ42 significantly decreased p75NTR expression in neuronally differentiated SH-SY5Y cells after 3 hr of treatment (Fig. 3A,B) but not after 24 hr of treatment (data not shown). This finding was consistent with those from the rat cortical cultures (Fig. 2). To evaluate Aβ-induced neuritic degeneration, we treated neuronally differentiated SH-SY5Y cells with Aβ42 for either 3 hr or 24 hr, then measured the levels of synapsin I (SynI), a recognized marker for nerve terminal and synapses. Aβ42 did not affect SynI protein levels after 3 hr of treatment (data not shown); however, Aβ42 significantly decreased SynI after 24 hr of treatment (Fig. 3C,D), indicating that neurites remain preserved when p75NTR expression is affected by Aβ.

Next, we examined whether neurotrophic factors could prevent, reverse, or ameliorate the neuritic degeneration caused by Aβ42. Becausee Aβ42 affected BDNF and p75NTR expression within 3 hr of treatment, we added to the cells at this time astrocyte-derived neurotrophic factors (NGF, BDNF, or GDNF), then assessed the effects of these factors on Aβ-related neuritic degeneration 24 hr after the initial Aβ42 treatment. We found that BDNF significantly preserved SynI levels in Aβ42-treated, neuronally differentiated SH-SY5Y cells, suggesting that BDNF prevented neuritic degeneration caused by Aβ (Fig. 3C,D). Although both NGF and GDNF also preserved SynI levels, BDNF was the most effective of the three neurotrophic factors (Fig. 3C,D). Moreover, immunocytochemical studies also confirmed that BDNF preserved the immunostaining pattern of synaptophysin (Syn) in Aβ42-treated, neuronally differentiated SH-SY5Y cells (Fig. 4).

DISCUSSION

In the present study, we used molecular biological and biochemical analyses to assess how Aβ influences the expression of neurotrophic factors by astrocytes. We found that astrocytes elevated BDNF expression and protein production at an early stage, within 3 hr of Aβ treatment (Fig. 1). Although several studies have already shown that Aβ induces immune responses and chemokine

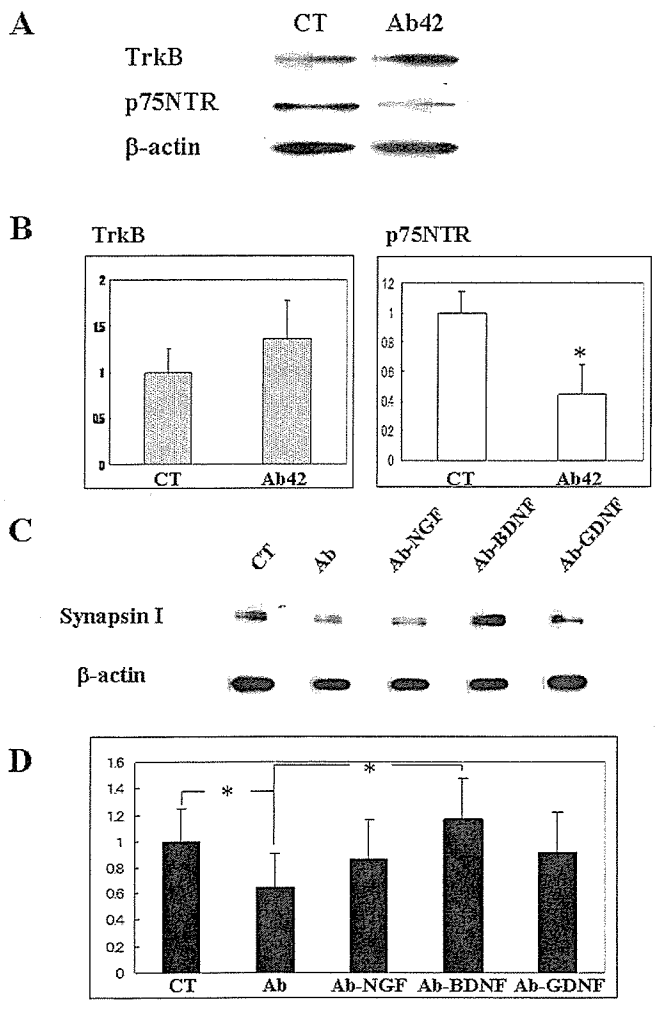

Fig. 3. **A:** Western blots showing the effect of Aβ on expression of TrkB and p75NTR in neuronally differentiated SH-SY5Y cell cultures. In this experiment, Aβ42 was diluted in culture medium to a final concentration of 10 μM. In neuronally differentiated SH-SY5Y cells, Aβ42 seemed to increase TrkB expression but reduced p75NTR expression after 3 hr of Aβ treatment. **B:** Histograms comparing the levels of TrkB and p75NTR protein expressed in neuronally differentiated SH-SY5Y cell cultures after 3 hr of Aβ treatment. Although the increase in TrkB expression was not significant, the decrease in p75NTR expression was statistically significantly. All data were normalized according to β-actin protein levels (control group; CT). Values are means ± SD. $*P < 0.02$. CT, control contained the same concentration of DMSO found in the Aβ treatments; Ab, Aβ42 treatment. **C:** Western blots showing that neurotrophic factors can rescue neuritic degeneration caused by Aβ42 toxicity. In this experiment, neuronally differentiated SH-SY5Y cell cultures were incubated with Aβ42 for 3 hr, then neurotrophic factors (NGF, BDNF, and GDNF) were added to the cultures, which were assessed 24 hr after the initial Aβ42 treatment by immunoblotting with anti-synapsin I antibody. **D:** Histograms comparing synapsin I protein levels expressed in neuronally differentiated SH-SY5Y cell cultures after Aβ treatment in the absence or presence of neurotrophic factors. In cultures treated with Aβ42 for 24 hr, Aβ42 significantly reduced synapsin I protein levels, suggesting that Aβ42 disrupted neurites (cf. C). BDNF treatment, however, significantly inhibited the Aβ42-associated neuritic degeneration. All data were normalized according to β-actin protein levels (control group; CT). Values are means ± SD. $*P < 0.02$. Ab, Aβ42 treatment without neurotrophic factors; Ab-NGF, NGF treatment given 3 hr after Aβ42 treatment; Ab-BDNF, BDNF treatment given 3 hr after Aβ42 treatment; Ab-GDNF, GDNF treatment given 3 hr after Aβ42 treatment.

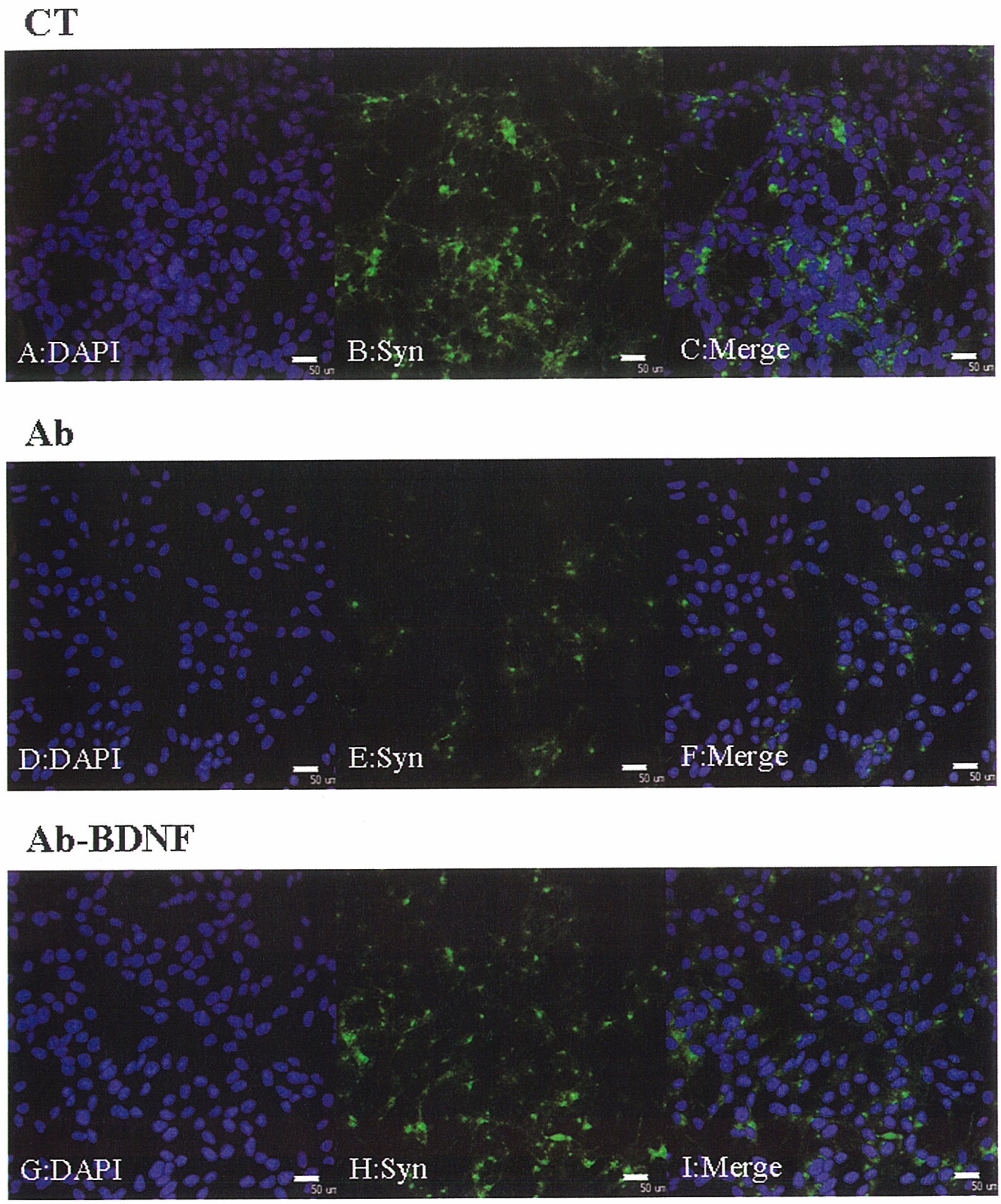

Fig. 4. Photomicrographs of neuronally differentiated SH-SY5Y cell cultures. In this experiment, neuronally differentiated SH-SY5Y cell cultures were incubated with Aβ42 for 3 hr, then BDNF was added to the cultures, which were assessed 24 hr after the initial Aβ42 treatment by immunocytochemistry with antisynaptophysin antibody and DAPI. **A–C:** In control group (CT), synaptophysin (Syn) was immunostained in almost all cells and neurites, suggesting that neurites were well preserved. **D–F:** In cultures treated with Aβ42 for 24 hr, Aβ42 clearly reduced Syn-immunoreactive cells and neurites, suggesting that Aβ42 caused neuritic degeneration. **G–I:** BDNF treatment preserved the immunostaining pattern of Syn, compared with Aβ42 treatment alone (D–F). CT, control contained the same concentration of DMSO found in the Aβ treatments; Ab, Aβ42 treatment without BDNF; Ab-BDNF, BDNF treatment given 3 hr after Aβ42 treatment; Syn, synaptophysin. Scale bars = 50 μm.

production in astrocytes (LaDu et al., 2001; Smits et al., 2002; Deb et al., 2003), our study is the first to show that astrocytes increase neurotrophic factor production in response to Aβ.

We found that Aβ42 induced a greater BDNF response from astrocytes than did Aβ40 (Fig. 1). This observation is not surprising, insofar as Aβ42 is considered to be the more toxic of the two Aβ species and is more closely associated with AD pathogenesis than is Aβ40 (Burdick et al., 1992; Jarrett et al., 1993; Suzuki et al., 1994; Younkin, 1994). We believe that astrocytes up-regulated BDNF production in response to Aβ toxicity. Interestingly, Aβ40 also significantly increased BDNF production from astrocytes (Fig. 1B). Insofar as Aβ40 is thought to be the primary form of Aβ found in normal brain (Haas et al., 1992; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993; Vigo-Pelfrey et al., 1993), the Aβ40-induced BDNF production from astrocytes that we observed may be one way in which astrocytes maintain normal brain homeostasis.

In primary cerebral cortical cultures after 3 hr of Aβ treatment, the same time frame during which expression of BDNF increased in astrocyte cultures (Fig. 1), expression of p75NTR significantly decreased (Fig. 2). These findings suggest that, relatively soon after exposure to Aβ, astrocytes produce BDNF, which in turn binds TrkB receptors but not p75NTR in neurons, thereby up-regulating neurotrophic signaling in neurons against Aβ rather than initiating apoptotic signaling (Huang and Reichardt., 2003; Nykjaer et al., 2005; Woo et al., 2005).

The most notable finding of the present study was that BDNF rescued neuronally differentiated human cells from neuritic degeneration caused by Aβ (Fig. 4). The fact that BDNF expression is decreased in AD brains (Phillips et al., 1991; Connor et al., 1997; Hock et al., 2000; Holsinger et al., 2000) may explain why neuritic degeneration and synaptic impairment occurring early on in AD fail to improve but, rather, progressively worsen to more advanced stages of AD pathology. It is now clear that neuronal synaptic degeneration occurs during early stages of AD (Masliah, 2001; Yao et al., 2003; Tsai et al., 2004; Reddy et al., 2005). Thus, inhibiting the progression of neuritic degeneration and synaptic impairment with BDNF would be key in therapeutically preventing mild cognitive impairment (MCI) from proceeding to AD.

In the present study, although we observed increased BDNF production from astrocytes and decreased p75NTR expression in neuronal cells fairly soon (3 hr) after Aβ treatment (Figs. 2, 3), we did not observe signs of drastic neuritic degeneration at this stage (data not shown). This situation may reflect what occurs during the early stages of MCI: Aβ initiates neuritic degeneration, and neurons respond to the BDNF released from astrocytes. This rapid response scenario is consistent with our observations that BDNF production was relatively constant 12 hr or 24 hr after Aβ treatment (data not shown), but neurites were significantly disrupted (Figs. 3,4). Our findings thus suggest that BDNF treatment (e.g., BDNF gene therapy) might be effective in staving off Aβ pathologies and/or synaptic degeneration before they become fully expressed. Moreover, because astrocytes are the source of the BDNF response against Aβ, astrocytes would be a prime target for MCI and AD therapies. Indeed, up-regulating the production of astrocytic BDNF in the brains of MCI patients may prove to be a conservative, yet effective, therapy for preventing MCI advancement, although a means for establishing a persistent BDNF production system in brain would be needed.

In summary, we found that astrocytes up-regulated BDNF production in response to Aβ in vitro and that BDNF treatment rescued Aβ-related neuritic degeneration. This will be a major breakthrough if it can be shown that BDNF treatment has a similar effect in vivo. Thus, we will continue our investigations of other astroglial functions in AD pathology with the aim of discovering more clues that can be translated into potential therapies for the devastation caused by AD.

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Rapid Communication

Ubiquitination of APOBEC3 proteins by the Vif–Cullin5–ElonginB–ElonginC complex

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Abstract

APOBEC3 proteins are antiviral host factors for a wide variety of retroviruses. HIV-1 Vif overcomes the antiviral activity of APOBEC3G by ubiquitinating the protein. In this study, we examined the ability of Vif to antagonize other family members of APOBEC3 proteins, together with its mechanism. Using HIV infectivity, virion incorporation, immunoprecipitation, and in vitro ubiquitin conjugation assays, we show that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by a Vif–Cullin5–ElonginB–ElonginC (Vif–BC–Cul5) complex. These results suggest that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

Keywords: HIV-1; Cytidine deaminase; Virion incorporation; Ubiquitin; Cullin5–ElonginB–ElonginC complex

Introduction

APOBEC3G (A3G) is a potent antiretroviral host factor (Sheehy et al., 2002). It deaminates cytidine to uridine in nascent minus-strand viral DNA, inducing G-to-A hypermutation in the plus-strand viral DNA (Harris et al., 2003; Mangeat et al., 2003; Shindo et al., 2003; Zhang et al., 2003). HIV-1 Vif protein overcomes the antiviral activity of A3G by targeting it for ubiquitin-dependent degradation (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). Vif interacts with cellular proteins, Cullin5 (Cul5), Elongin B (EloB), Elongin C (EloC), and Rbx1 through its novel SOCS-box motif to form a ubiquitin ligase (E3) complex (Vif–BC–Cul5) and functions as a substrate recognition subunit of the complex (Kobayashi et al., 2005; Mehle et al., 2004; Yu et al., 2003, 2004b). A3G belongs to the APOBEC superfamily of cytidine deaminases (Jarmuz et al., 2002), and several studies have reported that other members of this family such as APOBEC3B (A3B) and APOBEC3F (A3F) also have an antiviral activity on HIV-1, while the involvement of Vif in antagonizing these enzymes remains controversial (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). We have recently reported that the Vif–BC–Cul5 complex could indeed work as an E3 ligase by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 complex (Kobayashi et al., 2005). Using the assay, here, we show that the Vif–BC–Cul5 complex ubiquitinates A3F as well as A3G, but not A3B. We also demonstrate that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by the Vif–BC–Cul5 complex, indicating that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

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Results

We first tested the antiviral activity of A3G, A3B, and A3F on HIV-1 as well as their incorporation into HIV-1 virions. As shown in Fig. 1A, expression of APOBEC3 proteins suppressed the infectivity of ΔVif virions to various extents. HIV-1

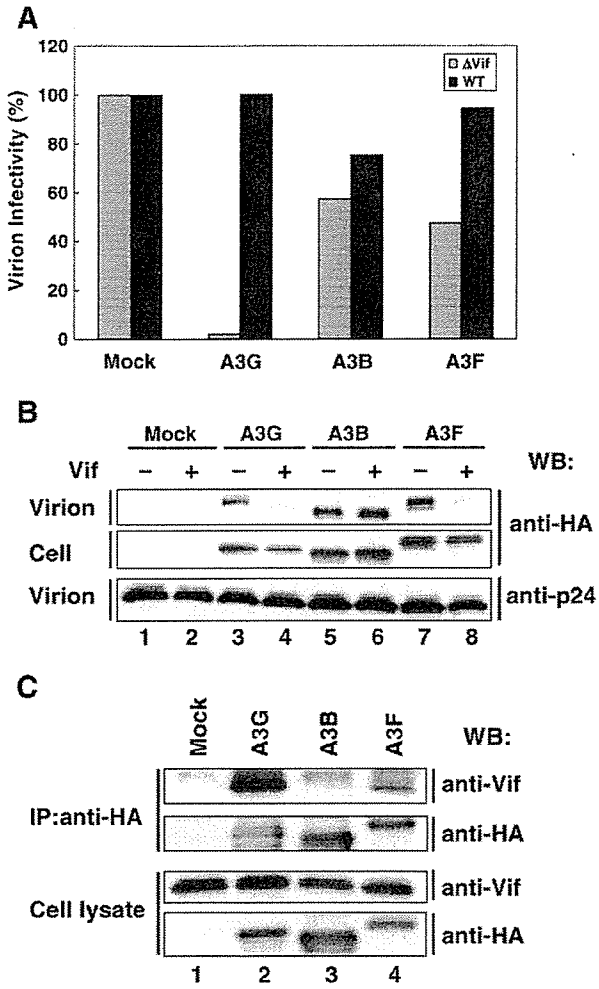

Fig. 1. Antiviral activity of APOBEC3 proteins on HIV-1. (A) A3B is resistant to HIV-1 Vif. We transfected HEK293T cells with pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G in the presence of pcDNA3/HA-based vectors (a mock, A3G, A3B, and A3F). Viruses from these cells were challenged to M8166 cells, and productive infection was measured by luciferase activity. Values are presented as the percent infectivity relative to the values of each virus without expression of APOBEC3 proteins. Expression of APOBEC proteins suppressed the infectivity of ΔVif virions, and HIV-1 Vif overcame the antiviral activity of A3F as well as A3G, but not that of A3B. (B) Vif inhibited virion incorporation of A3G and A3F, but not that of A3B. HIV-1 virions prepared as described above were precipitated by ultracentrifugation and subjected to immunoblot with anti-HA (top panel) and anti-p24 (bottom panel) mAbs. Cell lysates of producer cells were also subjected to immunoblot with anti-HA mAb (middle panel). (C) Vif could bind to A3G and A3F, but not to A3B. HEK293T cells were co-transfected with expression vectors for APOBEC3 proteins and Vif. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb (top panel) or anti-HA mAb (2nd top panel). Vif was coprecipitated with A3G and, to a lesser extent, with A3F, but not with A3B. Cell lysates were also subjected to immunoblot with anti-Vif mAb (3rd top panel) or anti-HA mAb (bottom panel).

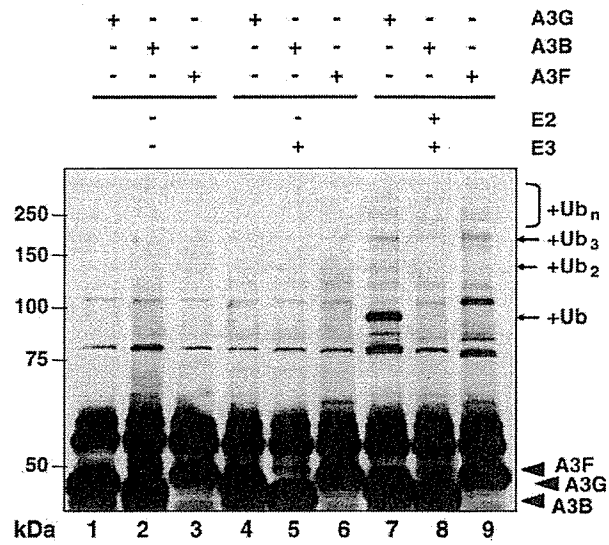

Fig. 2. In vitro ubiquitination of APOBEC3 proteins. An in vitro ubiquitin conjugation assay was performed as described in Materials and methods. GST-ubiquitin-conjugated A3G and A3F proteins were specifically detected as ladder (arrows) by immunoblotting with anti-HA mAb.

Vif overcame the antiviral activity of A3G and A3F, but not that of A3B, suggesting that APOBEC3B was resistant to HIV-1 Vif. The infectivity of the wild type (WT) virion was 8 to 47 times higher than that of ΔVif virion with A3G, and 2 to 3 times higher with A3F, suggesting that HIV-1 Vif antagonized A3G more effectively as compared to A3F. An immunoblotting of APOBEC3 proteins in virions and producer cells (Fig. 1B) revealed that all APOBEC proteins were incorporated into ΔVif virions (Fig. 1B, top panel, lanes 3, 5, and 7). An immunoblot with anti-β-actin mAb revealed no incorporation of this abundant cellular protein into virions, suggesting the specific incorporation of APOBEC3 proteins (data not shown). Vif could inhibit virion incorporation of A3G and A3F effectively (top panel, lanes 4 and 8, respectively), but not that of A3B (top panel, lane 6). In parallel, Vif reduced intracellular levels of A3G and A3F, but not that of A3B (Fig. 1B, middle panel), and reduction of the intracellular level of A3G by Vif was stronger than that of A3F. We next examined the physical interaction of Vif with APOBEC3 proteins by an immunoprecipitation assay. HIV-1 Vif was co-immunoprecipitated with A3G and, to a lesser extent, with A3F (Fig. 1C, top panel, lanes 2 and 4, respectively), but not with A3B (lane 3). These results suggested that Vif inhibited the virion incorporation of A3F as well as A3G, but not that of A3B because Vif could not bind to A3B. This also suggested that the ability of Vif to bind these proteins corresponded to the extent of reduction of their intracellular levels. Finally, we tested the E3 activity of Vif–BC–Cul5 ligase complex on APOBEC3 proteins by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 as previously reported (Kobayashi et al., 2005). As shown in Fig. 2, the Vif–BC–Cul5 (E3) complex specifically ubiquitinated A3G (lane 7) and, to a lesser extent, A3F (lane 9) since it did not ubiquitinate these when E2 was omitted. The magnitude of ubiquitination of these proteins corresponded to the extent to which Vif overcame the antiviral

activity of these proteins. In contrast, the assay showed no ubiquitination of A3B (lane 8).

Discussion

HIV Vif is known to antagonize the antiviral activity of A3G by excluding the protein from HIV virion, which is attributed to the ubiquitination of A3G by the Vif–BC–Cul5 complex as previously reported (Kobayashi et al., 2005). In this study, we show the clear correlation between the function of Vif to antagonize APOBEC3 proteins and the ubiquitination of these by Vif–BC–Cul5 ubiquitin ligase complex using the in vitro ubiquitin conjugation assay. Vif overcomes the antiviral activity of A3F by ubiquitinating it through the Vif–BC–Cul5 complex as reported with A3G although to a lesser extent. However, Vif cannot overcome the antiviral activity of A3B because it cannot bind to A3B. The magnitude of inhibitory activity of Vif against the proteins corresponds to the extent of ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex as well as the binding ability of Vif to APOBEC3 proteins. This suggests two possibilities. One is that the binding of Vif to APOBEC3 proteins might induce the changes in its conformation or subcellular localization leading to unpackaging into virions as reported by the Strebel laboratory (Kao et al., 2004) because the binding ability of Vif to APOBEC3 proteins correlates to the inhibitory activity on APOBEC3. The other is, as we reported previously, that the ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex is essential for Vif function against the proteins. Although we cannot fully exclude the former possibility at this time, we believe that the latter is more likely because our in vitro ubiquitination assay showed the clear correlation between the in vitro ubiquitination of APOBEC3 proteins and the inhibitory effect of Vif on the proteins. This could not be fully explained by the former mechanism alone. Further study is necessary to fully elucidate this mechanism.

The antiviral activity of A3B on HIV-1 has been controversial. Some groups reported a weak inhibitory effect of A3B on HIV-1 (Bishop et al., 2004; Yu et al., 2004a), while others recently reported a strong inhibition (Doehle et al., 2005). In this study, we found only a weak antiviral activity of A3B on HIV-1. By sequencing, we found some SNPs in the coding region of A3B according to National Center for Biotechnology Information database. Although we could not fully explain the discrepancies of the anti-HIV-1 activities of A3B among studies, one explanation might be that SNPs in the coding region of A3B might affect its antiviral activity. Further study on this matter is also be warranted.

We previously demonstrated that ubiquitination of A3G by the Vif–BC–Cul5 complex is essential for Vif function against A3G. In this study, we further extend this notion by showing that the ability of Vif to inhibit antiviral activity of APOBEC3 positively correlates with its ability to bind and ubiquitinate APOBEC3 by Vif–BC–Cul5. This will provide us with new insights into the mechanism of Vif function to antagonize APOBEC proteins and to identify new targets for therapeutic strategy.

Materials and methods

Plasmids and cell lines

Expression vector for hemagglutinin (HA)-tagged human A3G, pcDNA3/HA-A3G, was constructed as previously described (Kobayashi et al., 2004). pcDNA3/HA-A3F was constructed in the same way. pNLA1-43Vif was constructed by inserting a Vif fragment from NL4-3 into the subgenomic expression vector pNL-A1 (a kind gift from Dr. K. Strebel), which expresses all HIV-1 proteins except for *gag* and *pol* products (Strebel et al., 1987). pcDNA3/HA-A3B was a kind gift from Dr. K Imada (Kyoto University) (Hishizawa et al., 2005). pNL43/ΔEnv-Luc and pNL43/ΔEnvΔvif-Luc were constructed as previously described (Shindo et al., 2003). HEK293T and M8166 cells were maintained as previously described (Shindo et al., 2003).

Infectivity assay with luciferase reporter viruses

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G, A3B, and A3F by calcium phosphate method as previously described (Shindo et al., 2003). Productive infection was measured by luciferase activity. Values were presented as percent infectivity relative to the value of each virus without expression of APOBEC3 proteins.

Co-immunoprecipitation assay

To see protein–protein interaction in vivo, we performed an immunoprecipitation assay as described previously (Shindo et al., 2003). pcDNA3/HA-A3G, A3B, or A3F was co-transfected with pNLA1-43Vif into HEK293T cells by calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH 7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol), and complexes were immunoprecipitated with anti-HA monoclonal antibody (mAb) (12CA5) (F. Hoffmann-La Roche Ltd.) and protein A–Sepharose beads (Amersham Biosciences Corp., Piscataway, NJ) at 4 °C. The beads were washed with lysis buffer and analyzed on immunoblot with anti-HA mAb or anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) (Simon et al., 1995).

In vitro ubiquitin conjugation assay

In vitro ubiquitin conjugation assay was performed as previously described (Kobayashi et al., 2005). In brief, a Vif–BC–Cul5 complex was purified from insect cells and incubated with immunopurified HA-A3G, A3B, or A3F from 293T cells in reaction buffer containing E1, E2, GST-ubiquitin, NEDD8, Ubc12 (E2 for NEDD8), and APP-BP/Uba3 (E1 for NEDD8) at 37 °C for 1 h. Samples were subjected to

immunoblot to detect GST-ubiquitin-conjugated HA-APOBEC3 proteins.

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