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Alzheimer's Disease-linked Swedish Amyloid Precursor Protein Mutation Induces Cell Death by Increasing Reactive Oxygen Species Generation

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ABSTRACT **: The Swedish double mutation (KM670/671NL) of amyloid precursor protein (Swe-APP) is associated with early-onset familial Alzheimer's disease (FAD) and increases amyloid beta peptide production. Although APP/Aβ mediated neurotoxicity is observed both in vitro and *in vivo*, the relationship between mutant APP expression, Aβ production, and neuronal death observed in the brains of FAD patients remains to be elucidated. In this study, we investigated the mechanisms of Swe-APP-induced cell death in HEK293 and NGF-differentiated PC 12 cells. We found that the expression of Swe-APP induced cytochrome C relase, activation of caspase 3 in HEK 293 and NGF-differentiated PC 12 cells. We also show that the reactive oxygen species (ROS) was detected in Swe-APP expressing HEK 293 cells and NGF-differentiated PC 12 cells and that pretreatment with vitamine E attenuated the cellular death, cytochrome C release induced by Swe-APP expression, indicating the involvement of free radical in these processes. These results suggest one of possible apoptotic mechanisms of Swe-APP which could occur through cytochrome C release from mitochondria and this apoptosis inducing effects could be at least in part, due to ROS generation by Swe-APP expression.**

Key words : **Alzheimer's disease, Swedish Amyloid Precursor Protein, Reactive Oxygen Species, Cytochrome C, Apotosis**

Introduction

Aβ, a major component of amyloid plaques, one of the histopathological hallmarks of Alzheimer's disease (AD), produced by aberrant processing of the amyloid precursor protein (APP) has been postulated to be a causal factor in the pathogenesis of AD (Suh and Checler, 2002). Several mutations have been detected within APP gene in familial Alzheimer's disease (FAD) (Selkoe *et al.*, 2001). Among them, a double mutation preceding the N-terminus of the Aβ domain (KM595/596NL, numbered based on APP 695), initially identified in a Swedish family (Swe-APP) is the most popular form of the FAD-related mutations (Suh and Checler, 2002). Swe-APP mutation is associated with three to sixfold increase in total Aβ production by enhancing cleavage by β-secretase, which involves $A\beta_{1-40}$ as well as $A\beta_{1-42}$.

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Although APP/Aβ mediated neurotoxicity is observed both in vitro (Mattson *et al.*, 1992) and *in vivo* (Kowall *et al.*, 1992), the relationship between mutant APP expression, Aβ production, and neuronal death observed in the brains of FAD patients, and the underlying mechanisms leading to the massive neurodegeneration are still not understood (Eckert *et al.*, 2001).

There have been many reports showing that much evidence of oxidative stress manifested by protein oxidation, lipid peroxidation, DNA oxidation and 3-nitrotyrosine formation, is observed in AD brain (Butterfield, 2002). In this study, we investigated the mechanisms by which Swe-APP exerted cytotoxicity in in HEK293 cells and NGF-differentiated PC12 cells. We examined whether reactive oxygen species (ROS) was involved in the cytotoxic effects of Swe-APP and found increase in ROS accumulation in Swe-expressing cells.

Recent studies show that multiple death signals converge on the mitochondria (Wei *et al.*, 2001).

Damaged mitochondria release proapoptotic factors including apoptosis inducing factor (AIF) and cytochrome C (cyt C) into the cytosol, which can activate procaspase-9 and the caspase-3 with the help of Apaf-1 and dATP. Cyt C release can be regulated by various factors including free radicals, increased $[Ca]^{+2}$ concentration, inducing mitochondrial membrane permeability transition, and also by members of the Bcl-2 family, including Bcl-2, Bcl-xl and Bad (Yang *et al.*, 1997).

In this study, we investigated the mechanisms by which Swe-APP exerted cytotoxicity in HEK 293 and differentiated PC 12 cells and demonstrated that Swe-APP induced cyt C release, caspase 3 activation, and that these effects were attenuated by pretreatment with vitamine E.

Materials and Methods

Reagents

Anti-cytochrome c antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase 3 and anti-activated caspase 3 antibodies were from Transduction Laboratories (Lexington, KY). Complete mixture of protease inhibitors was from Roche Molecular Biochemicals. Anti-mouse HRP linked antibodies were from Amersham Pharmacia Biotech.

Plasmid construction and expression

Wt-APP 695 and Swe-APP695 cDNA were kind gifts from Dr. Sisodia at the University of Chicago. Plasmid pSwe-APP (Lo *et al.*, 1994) encodes Myc epitope-tagged human APP-695 that harbors the Swedish-FAD-specific amino acid substitutions (K595N and M596L).

Cell line, cultured conditions and transfection

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with heat-inactivated 10% fetal bovine serum and 0.3% antibiotics at 37°C. PC12 cells were treated with NGF (50 ng/ml; Calbiochem, Darmstadt, Germany) to differentiate for 48 h. Cells were transiently transfected using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Immunocytochemistry

Cells were incubated in 37°C pre-warmed serum-free Dulbecco's modified Eagle's medium loaded with the Mitotracker-Red (Molecular Probes) at 37°C. Then cells were fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 30 min at room temperature. After quenching in 50 mM NH_4Cl-PBS for 10 min, cells were washed in PBS and permeabilized for 30 min at room temperature in PBS containing 0.1% Triton X-100 and 1 mg/ml bovine serum albumin (permeabilization buffer). Cells were incubated with primary antibodies for 1 h. After three washes, primary antibodies were revealed by incubating the cells for 45 min with FITC or $Cy^{TM}3$-conjugated secondary antibodies. After three washes in permeabilization buffer and a wash in PBS, cells were mounted on microscope slides in mounting medium (DAKO). Confocal microscopic observation was performed using a Bio-Rad MRC 1024. For the detection of active caspase-3, fluorescent mounting medium (DAKO) including DAPI was used for the labeling of nucleus.

Western blotting

Cells were lysed in a lysis buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1% Triton, 0.5% SDS, 0.5% DOC, and protease inhibitors. For detection of active caspase 3, 50 μg of cell lysate was resolved by SDS polyacrylamide gel electrophoresis (18%).

For detection for cyt C in mitochondrial and cytosolic fraction, after cell lysis, the mitochondrial and cytosolic fraction were obtained according to the previous method (Kang *et al.*, 1995). Cytosol (50 μg) and mitochondria (50 μg) were resolved by SDS polyacrylamide gel electrophoresis (15%), blotted onto a nitrocellulose membrane (pore size, 0.2 μm) and identified with primary and secondary antibodies (Santa Cruz). The immunoblotting signal was visualized with an ECL kit (Amersham).

Evaluation of apoptosis with TUNEL staining

TUNEL staining was performed according to the manufacturer's protocol (In situ cell death detection kit; Roche Diagnostics GmbH, Germany). The number of TUNEL positive cells in four random fields was counted in two sets of experiments, and the results were normalized as percentage ratios compared with the total number of the cells transfected with wt-APP and Swe-APP.

Cell viability

Cell viability of transiently transfected with wt- or Swe-APP HEK 293 cells or differentiated PC 12 cells

were assessed by XTT and LDH assay according to the manufacturer's instructions.

Measurement of ROS generation

Cells in 6 well or 96 well plates were washed with HEPES-buffered saline and incubated in the dark for 1 h in HBS containing 200 μM of dichloro-2,7-fluorescein diacetate (DCFHDA) (Fluka, St. Gallen, Switzerland). ROS generation was detected as a result of DCFH oxidation. Cells in 6well plates were then examined using fluorescence microscopy. Fluorescence in cells in 96well plates was read immediately at wavelengths of 485nm for excitation and 530 nm for emission with a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data is expressed as mean±SEM values. ANOVA tests were applied to study the relationship between the different variables. $p < 0.05$ was considered to be significant.

Results

At first, we investigated whether wt- or Swe-APP induced apoptosis and decreased cell viability in HEK293 cells and NGF-differentiated PC12 cells. Significant staining with terminal deoxynucleotidyl transferase (TUNEL) was shown in Swe-APP transfected HEK293 cells (Fig. 1A) and differentiated PC12 cells (data not shown), compared to the mock-or wt-APP transfected cells. TUNEL-positive HEK 293 cells 48 h after transfection were 4.1±0.2%, 15.0±3.0% or 38.0 ±4.0% in mock transfected, wt-APP or Swe-APP transfected cells, respectively. We also found that treatment with vitamine E at 50 μM for 48 h after transfection significantly reduced apoptotic cells observed in Swe-APP expressing cells (Fig. 1A), suggesting that ROS is involved in apoptosis induced by Swe-APP expression.

At 48 h post-transfection, HEK293 cells expressing wt-APP or Swe-APP decreased cell viability to 95.1± 12.1% or 58.5±9.7%, respectively, compared to the mock-transfected groups by XTT assay (data not shown). In NGF-differentiated PC 12 cells, mock, wt- or Swe-APP transfected cells showed 9.1±2.4%, 19.8±1.9% or 29.4±3.2% of LDH release compared to positive controls treated with 1% Triton X-100 (Fig. 1B). Treatment with vitamine E at 50 μM for 48 h after transfection significantly reduced the cell death induced by Swe-APP expression, indicating the free radical is involved in the cell death induced by Swe-APP (Fig. 1B). The results of Fig. 1A and B are consistent with the previous results by others that cell death induced by Swe-APP was attenuated by the use of vitamine E (Eckert *et al.*,

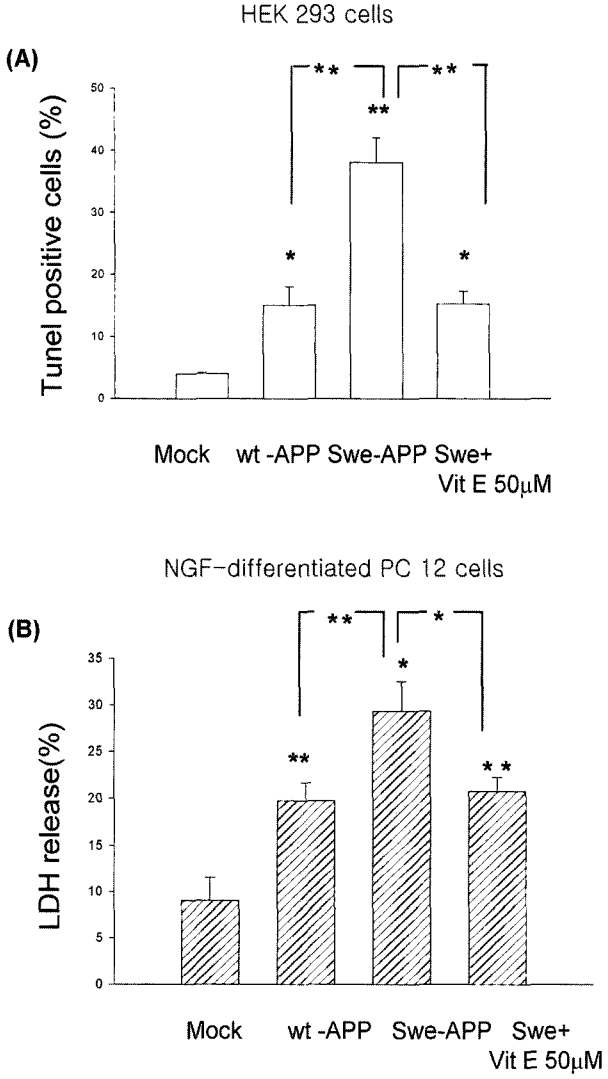

Fig. 1. Expression of Swe-APP exerts cytotoxicity in HEK 293 and NGF differentiated PC12 cells. (A) The percentage of TUNEL positive HEK 293 cells 48 h after transfection is shown in the graph. Data represents the mean±SEM of 4 separate experiments. (* $p < 0.05$, ** $p < 0.01$ by ANOVA). (B) The cell viability of mock, wt or Swe-APP transfected PC12 cells were examined at 48 h post-transfection by LDH assay. The results were expressed as percentages of LDH release obtained from 1% Triton-X 100 treated PC12 cells. Data represent mean±SE of 8-16 samples (*$p < 0.05$, *$p < 0.01$ by ANOVA).

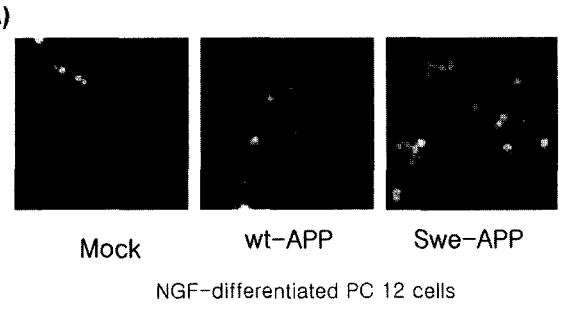

Fig. 2. Expression of Swe-APP induces ROS generation in HEK 293 and NGF differentiated PC12 cells. (A) ROS was detected using DCFHDA dye in NGF-differentiated PC 12 cells transfected with mock, wt-APP or Swe-APP with fluorescence miscroscopy. ROS was detected using DCFHDA in NGF-differentiated PC12 cells (B) and HEK 293 cells (C) transfected with mock, wt-APP or Swe-APP. Fluorescence in cells in 96well plates was read immediately at wavelengths of 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

2001).

Free radical was known to induce cyt C release by causing mitochondrial permeability transition (Kikuchi *et al.*, 2003). We examined whether expression of wt- or Swe-APP induced ROS generation using DCFHDA in HEK 293 cells and differentiated PC 12 cells and found that ROS generation in Swe-APP expressing cells was significantly higher than that of mock or wt-APP expressing cells (Fig. 2A. B, C). We also confirmed that treatment with vitamine E at 50 μM for 48 h after transfection significantly reduced the ROS generation induced by Swe-APP expression (Fig. 2 B and C).

Next, we examined cyt C release and presence of active caspase 3 in wt- or Swe-APP expressing HEK 293 cells and NGF-differentiated PC 12 cells at 48 h post-transfection (Fig. 3). Increased release of cyt C release into cytoplasm was found in Swe-APP expressing cells than in wt APP transfected HEK 293 cells (Fig. 3A, B and C). Yellow-color merged image indicates the location of cyt.C in mitochondria whereas released cyt C is visualized with a distinct green-colored image. Mitochondria are shown as red color (Fig. 3 A, B and C). Immunoblot results also showed the cyt C increase in cytosolic fraction and the decrease in mitochondrial fraction in differentiated PC 12 cells expressing Swe-APP (Fig. 3D). Active caspase 3 was detected in Swe-APP expressing PC 12 cells (Fig. 3E).

Vitamine E was known to be a scavenger of peroxyl radicals, and it is probably the most important inhibitor of the free-radical chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1999). To examine the effects of ROS in Swe-APP transfected cells, we pretreated 50 μM vitamine E after transfection and measured the level of cyt C in in cytosolic fraction. We found that the pretreatment with vitamine E significantly decreased the cyt C release induced by Swe-APP (Fig. 3D).

Discussion

Swe-APP, a double mutation preceding the N-terminus of the Aβ domain (KM595/596NL numbered based on APP 695), the most commonly found FAD genotype, has been known to increase total Aβ by enhancing the β-secretase activity (Selkoe *et al.*, 2001). However, the relationship between mutant APP expression, Aβ production, and neuronal death observed in the brains of FAD patients, and the underlying mechanisms leading to the massive neurodegeneration remains to be elucidated

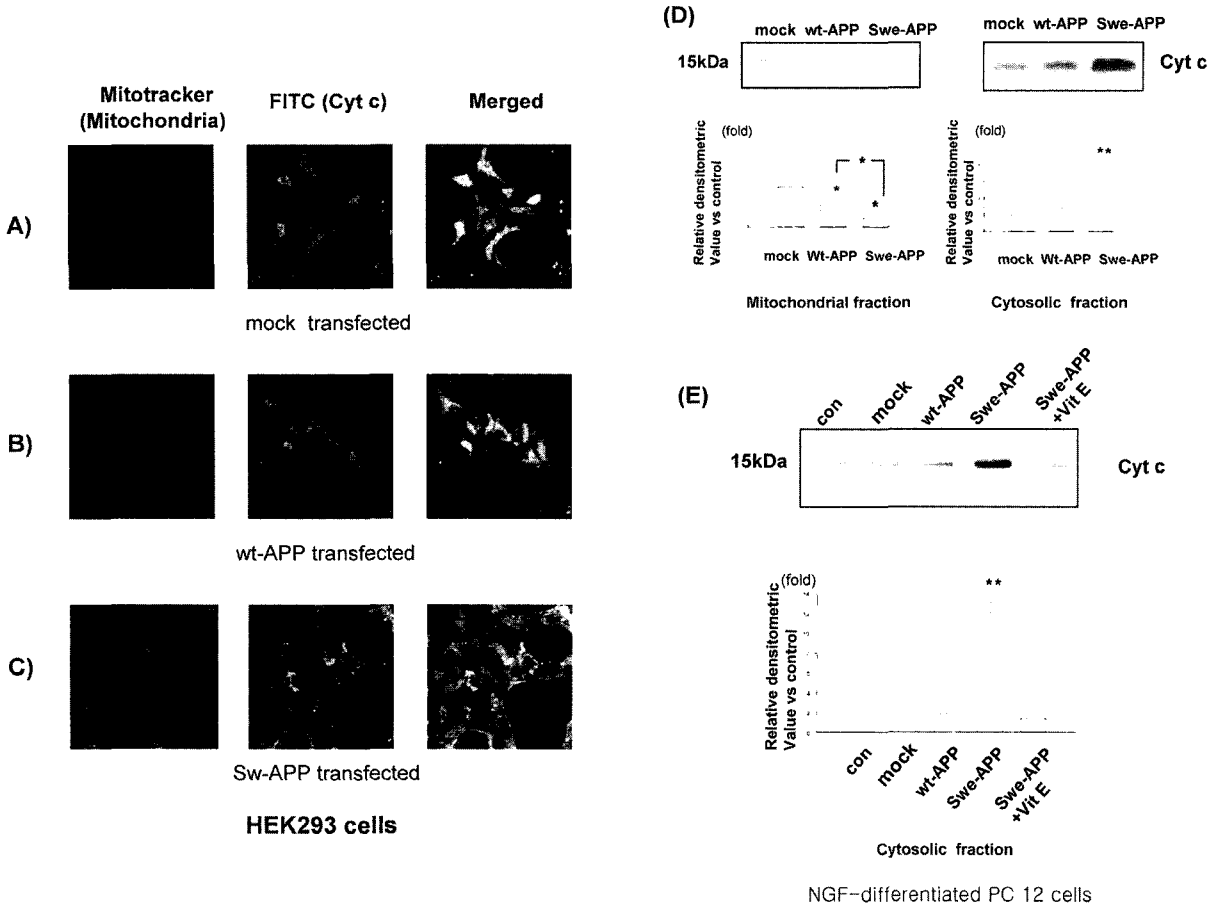

Fig. 3. Detection of cyt C release and active caspase-3 in wt or Swe-APP transfected HEK 293 and differentiated PC12 cells.
(A) (B) (C) Staining with mitotracker (red) and FITC-conjugated secondary antibody (green) represents the mitochondria and cyt C, respectively. (D) Representative immunoblots for the cyt C in mitochondrial or cytosolic fraction of wt- or Swe-APP transfeted PC 12 cells and densitometric values were shown. The results are representative of three separate experiments performed with different samples. (E) Representative immunoblots for the active caspase 3 of wild type or Swe- APP transfected PC 12 cells and densitometric values were shown. The results are representative of three separate experiments performed with different samples

(Eckert *et al.*, 2001).

In this study, we investigated the cytotoxic mechanisms of Swe-APP in HEK293 cells and NGF-differentiated PC12 cells. We demonstrated that expression of Swe-APP induced cyt C release, activation of caspase 3 (Fig. 3 A-E). In addition, we found that ROS generation was increased in Swe-APP transfected HEK 293 and differentiated PC 12 cells using DCF-DA dye (Fig. 2A-C). Much evidence of oxidative stress manifested by protein oxidation, lipid peroxidation, DNA oxidation and 3-nitrosine formation, has been observed in AD brain (Butterfield, 2002). Although the mechanism of Aβ-associated free radical formation is not fully understood, Aβ is believed to contact or insert into the neuronal and glial bilayer membrane and generate oxygen-dependent free radicals that then cause lipid peroxidation and protein oxidation (Suh and Checler, 2002).

Free radical is known to induce cyt C release by inducing mitochondrial permeability transition, activating procaspase 9 and the caspase-3 with the help of Apaf-1 and dATP and leads to apoptosis. In addition, free radical has been known to cause ER stress (Rao *et al.*, 2001). ER stress induces the processing of caspase-12 and also the upregulation of procaspase 12 has been reported in brefeldin A treated rat primary cortical neurons (Kikuchi *et al.*, 2003). Although downstream events following caspase 12 activation have not been reported up to now, a recent paper reported that procaspase 9 was a substrate for caspase 12 (Morishima

et al., 2002) suggesting that caspase 12 can activate caspase 9 without involvement of cyt C (Morishima *et al.*, 2002). It is reported that ER and mitochondria were crosstalking thorugh BAD and Bcl-2 regulation in rat primary cortical neurons (Elyaman *et al.*, 2002). Meanwhile, the cytotoxicity of Aβ has been reported to be mediated by ER-specific apoptosis via caspase 12 (Nakagawa *et al.*, 2000). Further study is needed on the effects of Swe-APP on ER-mediated apoptotic pathways, as ROS is well known to function as a ER stressor.

In this study, we investigated the mechanisms by which Swe-APP exerted cytotoxicity and demonstrated that Swe-APP induced apoptosis by mitochondria-mediated pathways and this effect may be derived from ROS generated by Swe-APP expression.

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