P. Riederer, H. Reichmann,
M. B. H. Youdim, M. Gerlach (eds.)

Parkinson's Disease and Related Disorders

Journal of Neural Transmission
Supplement 70

SpringerWienNewYork

J Neural Transm (2006) [Suppl] 70: 125–132

The effect of neuromelanin on the proteasome activity in human dopaminergic SH-SY5Y cells

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Summary. In Parkinson's disease (PD), the selective depletion of dopamine neurons in the substantia nigra, particular those containing neuromelanin (NM), is the characteristic pathological feature. The role of NM in the cell death of dopamine neurons has been considered either to be neurotoxic or neuroprotective, but the precise mechanism has never been elucidated. In human brain, NM is synthesized by polymerization of dopamine and relating quinones, to which bind heavy metals including iron. The effects of NM prepared from human brain were examined using human dopaminergic SH-SY5Y cells. It was found that NM inhibits 26S proteasome activity through generation of reactive oxygen and nitrogen species from mitochondria. The mitochondrial dysfunction was also induced by oxidative stress mediated by iron released from NM. NM accumulated in dopamine neurons in ageing may determine the selective vulnerability of dopamine neurons in PD.

Abbreviations

DMSO dimethyl sulfoxide, *DTT* dithiothreitol, *ECF* enhanced chemofluorescence, *MEM* minimum essential medium, *NM* neuromelanin, *PBS* phosphate-buffered saline, *SOD* superoxide dismutase, *PD* Parkinson's disease, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *UPS* ubiquitin-proteasome system, *ZsGFP* a green fluorescence protein homologue from Zoanthus sp.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorders in the aged and recently several genes responsible for the familial PD have been reported. However, the etiology of sporadic PD is still an enigma, and oxidative stress, impairment of mitochondrial function and the ubiquitin-proteasome system (UPS) are suggested to initiate a "malignant cycle" resulting in the cell death (Dexter et al., 1994). Especially, recent evidences suggest that failure of the UPS leads to aggregation and accumulation of abnormal proteins to form the inclusion bodies and induce neuronal cell death in the familial case of PD (Polymeropoulos et al., 1997; Shimura et al., 2000; Leroy et al., 1998). Dysfunction of the UPS is found to occur also in the sporadic form of PD also

(McNaught and Olanow, 2003). However, the factors, which determine the selective vulnerability of dopamine neurons are still unknown. One possibility is the involvement of dopamine and the metabolites themselves. Dopamine is a highly reactive catecholamine and is oxidized enzymatically and non-enzymatically. Autoxidation of dopamine produces speroxide and quinones, and polymerized quinones are interacted with proteinacious components and lipids (Gerlach et al., 1995; Dzierzega-Lecznar et al., 2004; Fedorow et al., 2005), to produce highly aggregated conjugates, neuromelanin (NM).

NM is a dark pigmented granule, which accumulate in catecholaminergic neurons of the substantia nigra and locus ceruleus. NM has been supposed to be a mere "waste box" in the neurons, but recently it was found to function as a reservoir of trace metals, such as iron (Zecca et al., 2001a). The involvement of iron in dopaminergic degeneration during ageing has been suggested repeatedly and recent studies show the content of iron in the substantia nigra was higher than that in the locus ceruleus (Zecca et al., 2004). Iron possibly induces cell death in nigral dopamine neurons through oxidative stress, as confirmed by studies on postmortem parkinsonian brains and on the cellular and animal PD models (Jenner, 2003). In the substantia nigra of PD brains, increased oxidative stress is suggested by increase in oxidative modification of lipids, proteins and DNA (Yoritaka et al., 1996). These results suggest that the effect of NM and iron should be examined on mitochondrial dysfunction, oxidative stress and the UPS.

In this paper we report the effects of NM prepared from control human brain on the production of reactive oxygen and nitrogen species (ROS, RNS) in mitochondria, the UPS activity using neuroblastoma SH-SY5Y cells. The in situ ubiquitin-26S proteasome activity was examined using the cells transfected with a proteasome sensor vector. The results are discussed in relation to the role of NM in selective vulnerability of dopaminergic neurons in PD and ageing.

Materials and methods

Materials

NM was isolated and purified from the substantia nigra of control human brains, as described previously (Gerlach et al., 1995) and was dissolved in distilled water containing 15 mM L-cysteine and 10% dimethyl sulfoxide (DMSO) (L-Cyst-DMSO solution) to be 0.5 mg/ml in the final concentration (Shamoto-Nagai et al., 2004). L-Cysteine was purchased from Sigma (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA), deferoxamine mesylate (DFX) and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma (St. Louis, MO, USA), and anti-polyubiquitin monoclonal antibody from NBT (Tokyo, Japan). A proteasome sensor vector, pZsProSensor-1, was purchased from BD Biosciences (Palo Alto, CA, USA). 2′,7′-Dichlorofluorescein (DCF), N-acetyl cysteine (NAC), L-cysteine and catalase from bovine liver, minimum essential medium (MEM) and other reagents were from Wako (Kyoto, Japan).

Measurement of in situ 26S proteasome activity in SH-SY5Y cells expressing a proteasome sensor vector

SH-SY5Y cells were cultured as reported (Shamoto-Nagai et al., 2004). Transfectant with a proteasome sensor vector was established using a pZsProSensor-1 eukaryotic expression vector, designed to express ZsGFP fused to the degradation domain of mouse ornithine decarboxylase, a specific substrate for 26S proteasome, by lipofection technique as reported previously (Shamoto-Nagai et al., 2004). The culture medium was changed with the medium containing L-Cyst-DMSO solution without (control) or with NM and the cells were cultured for 3 days. In addition, the cells transfected with proteasome sensor vector was incubated with various concentrations of iron in the presence or absence of 25 μM DFX or antioxidants for 20 h. The fluorescence of ZsGFP in the living cells was measured as described before (Shamoto-Nagai et al., 2004), and the fluorescence intensity of the cells was expressed as arbitrary fluorescence unit/mg protein. The protein amount was measured according to Bradford (1976).

Detection of polyubiquitinated proteins in the cells treated with NM

After treatment with NM for 1 or 3 days, the cells were gathered, washed with PBS, and lyzed in the RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Fifty μg of protein was subjected to SDS-polyacrylamide electrophoresis

using 12.5% polyacrylamide gel (Bio Craft, Tokyo, Japan), and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Plolyubiquitinated proteins were visualized using anti-polyubiquitin antibody and enhanced chemofluorescence (ECF) Western blotting kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously (Shamoto-Nagai et al., 2003).

Isolation of mitochondria from SH-SY5Y cells

Mitochondria were prepared from SH-SY5Y cells according to Desagher et al. (1999). The cells were gathered, washed with PBS and suspended in the isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.5) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannhein, Germany). The mitochondrial fraction was prepared by homogenization and two steps of centrifugation.

Measurement of ROS-RNS with H_2DCFDA

The mitochondria were suspended in PBS and the production of ROS-RNS was quantified fluorometrically by measuring DCF produced from H_2DCFDA (Crow, 1997). H_2DCFDA was added to be 50 μM to the mitochondria suspension in the presence or absence of NM suspension (1–5 μg/ml) in dark at 37°C. The increase in DCF fluorescence at 504 nm with excitation at 520 nm was measured at every 30 min for 3 h in a RF-5000 spectrofluorometer (Shimadzu, Kyoto, Japan). The generation of ROS-RNS was expressed as mol DCF per min per mg protein. The effects of DFX and anti-oxidants were also examined in the same way, after 15 min pre-incubation with DFX or the antioxidants.

Statistics

Experiments were repeated at least 3 times. The data was expressed as mean ± SD and the difference was evaluated by analysis of variance (ANOVA) followed by Scheffe's F-test. A p value less than 0.05 was estimated to be statistically significant.

Results

The effect of NM on in situ 26S proteasome activity

After treatment with NM or iron the accumulation of ZsGFP was observed in cytoplasm by fluorescence microscopy (Fig. 1). After 3 days' incubation, the fluorescence intensity in the cells treated with 0.1 μg/ml

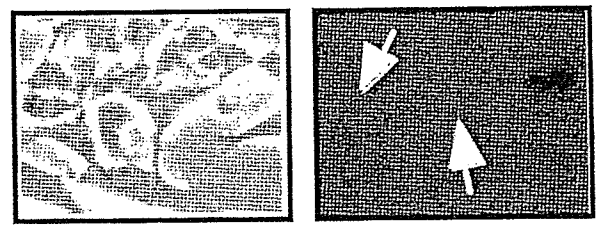

Fig. 1. Effects of NM on the in situ activity of 26S proteasome in SH-SY5Y cells transfected with the proteasome sensor vector. The cells were cultured in the presence of 0.1 μg/ml of NM for 3 days. Left: morphological observation of the cells treated with NM. Right: Accumulation of ZsGFP (white arrows) was observed by fluorescence microscopy

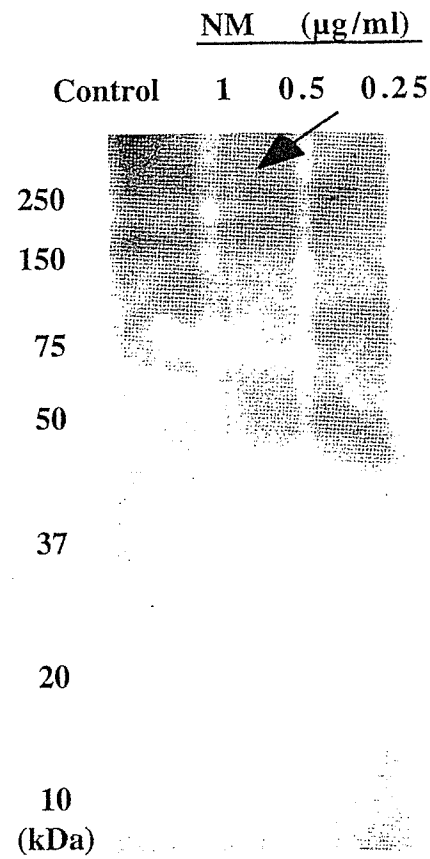

Fig. 2. Accumulation of poly-ubiquitinated proteins in SH-SY5Y cells treated with NM for 3 days (arrow). The cells were cultured with or without NM and lysed, then proteins were separated by SDS-PAGE and immunoblotted using anti-polyubiquitin antibody as described in the Materials and methods

NM increased to be that of 161% of control cells. The accumulation and the increase of fluorescence intensity was confirmed by measuring its fluorescence intensity by Fluorospectrometry also (Fig. 4). The increase of ZsGFP, that reflects the reduced activity of 26S proteasome by iron, was restored by DFX.

Accumulation of polyubiquitinated proteins in SH-SY5Y cells treated with NM

The cells treated with various concentration of NM was gathered and applied to Western blotting using anti-polyubiquitin antibodies. As shown in Fig. 2, in the cells treated with 1 μg/ml of NM, the accumulation of polyubiquitinated proteins, which is the specific substrate for 26S proteasome, was detected (arrow).

Increase of ROS-RNS in isolated mitochondria by NM

The production of $^{\bullet}OH$, NO and $ONOO^-$ in mitochondria was quantified fluorometrically using DCF cleaved from H_2DCFDA as an indicator (Fig. 2). In the presence of mitochondria, NM increased DCF fluorescence. In addition, SOD, but not catalase, reduced DCF production in mitochondria themselves. DFX significantly reduced the DCF production from mitochondira enhanced by NM. These results indicate that $O_2^{\bullet -}$ plays the key role in ROS-RNS production and the involvement of iron in the ROS-RNS production by NM.

Discussion

In this paper, it was clearly shown that NM increased ROS and RNS generation especially superoxide, in mitochondira, which

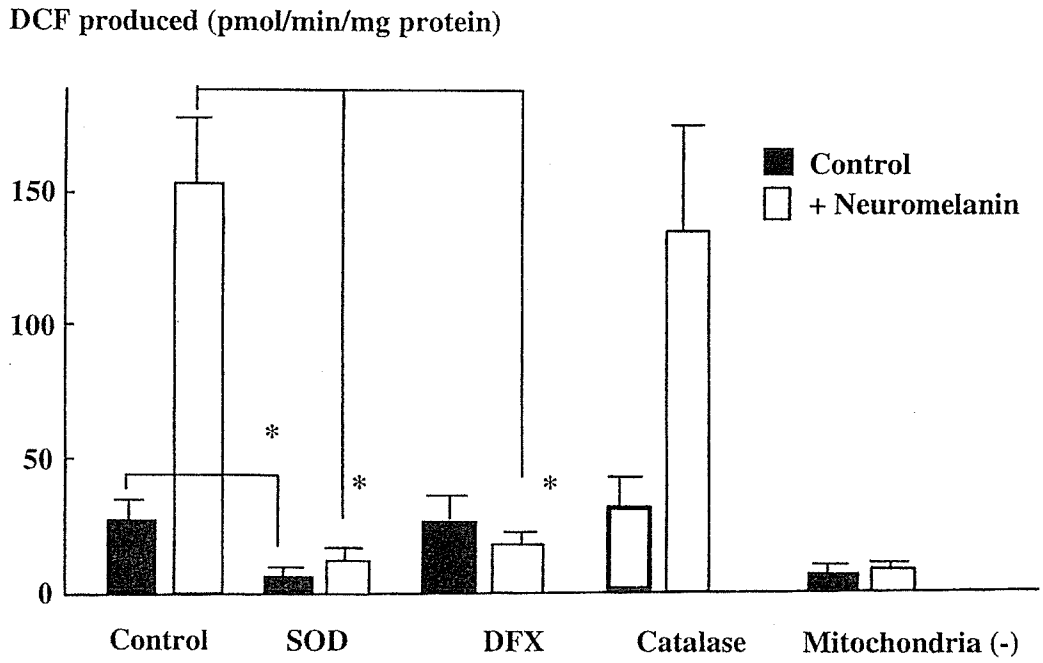

Fig. 3. Effects of SOD, deferoxamine mesylate (DFX), and catalase on DCF production in mitochondria prepared from SH-SY5Y cells. Mitochondria (30 μg protein) were incubated with or without 2.5 μg/ml NM in PBS, and the effects of SOD (1000 units), DFX (1 μM), and catalase (500 units) were examined. Generated ROS-RNS was quantitatively measured as DCF produced from H_2DCFDA and expressed as pmol/hr. The column and bar represent the mean and SD of triplicate measurements of 3 independent experiments. $^*P<0.01$

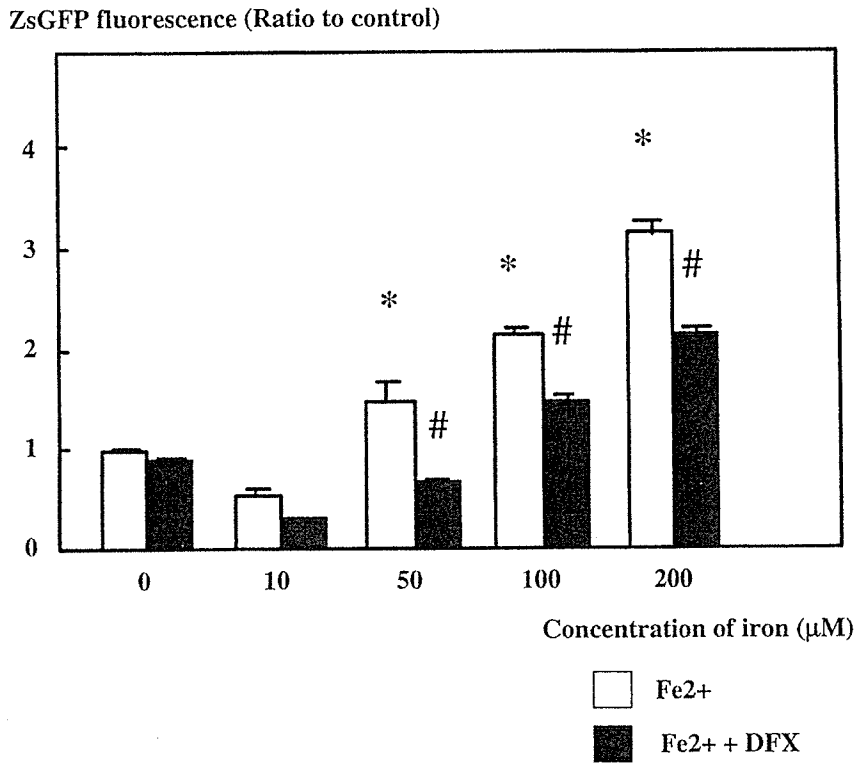

Fig. 4. Iron inhibited in situ activity of 26S proteasome in SH-SY5Y cells transfected with a proteasome sensor vector. After the treatment with iron with or without deferoxamine mesylate (DFX) for 20 h, the fluorescence intensity of ZsGFP, which is coded by a proteasome sensor vector, at 505 nm with excitation at 493 nm was quantified and expressed as arbitrary fluorescence unit/mg protein. The column and bar represent mean and SD of 3 experiments. After the treatment with iron, the fluorescence intensity increased in a dose-dependent manner. $^{*}p<0.05$ compared to the control. This increase was suppressed by 25 μM DFX significantly. $^{\#}p<0.05$ compared to the cells treated with iron without DFX

was mediated by released iron, as shown by the complete suppression of DFX. The onset of oxidative stress may deteriorate the function of mitochondria, in addition to the direct inhibition of mitochondrial respiratory chain enzymes. NM reduced the in situ activities of 26S proteasome, as shown using a green fluorescent protein homologue targeted to 26S proteasome. The mitochondrial toxins, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), were reported to induce neuronal death selective to dopamine neurons with formation of Lewy body-like inclusion body in in vivo models of PD (Betarbet et al., 2000; Kowall et al., 2000). The mechanism of cell death has not been elucidated, but the involvement of reduced UPS activity was suggested. Recently we found that mitochondrial dysfunction caused by rotenone, a complex I inhibitor, increased abnormal oxidative modification of proteins with acrolein, and reduced the activity of proteasome, through binding of aggregated oxidized protein to the catalytic site of 20S proteasome and direct adduction of acrolein to 20S protosome itself (Shamoto-Nagai et al., 2003). It may be reasonable to assume that mitochondrial dysfunction plays a central role in the pathogenesis of sporadic PD, and impairment of the UPS may be a final executor in the neural degeneration.

Iron is known to induce oxidative stress by enhancing electron transfer in a Fenton

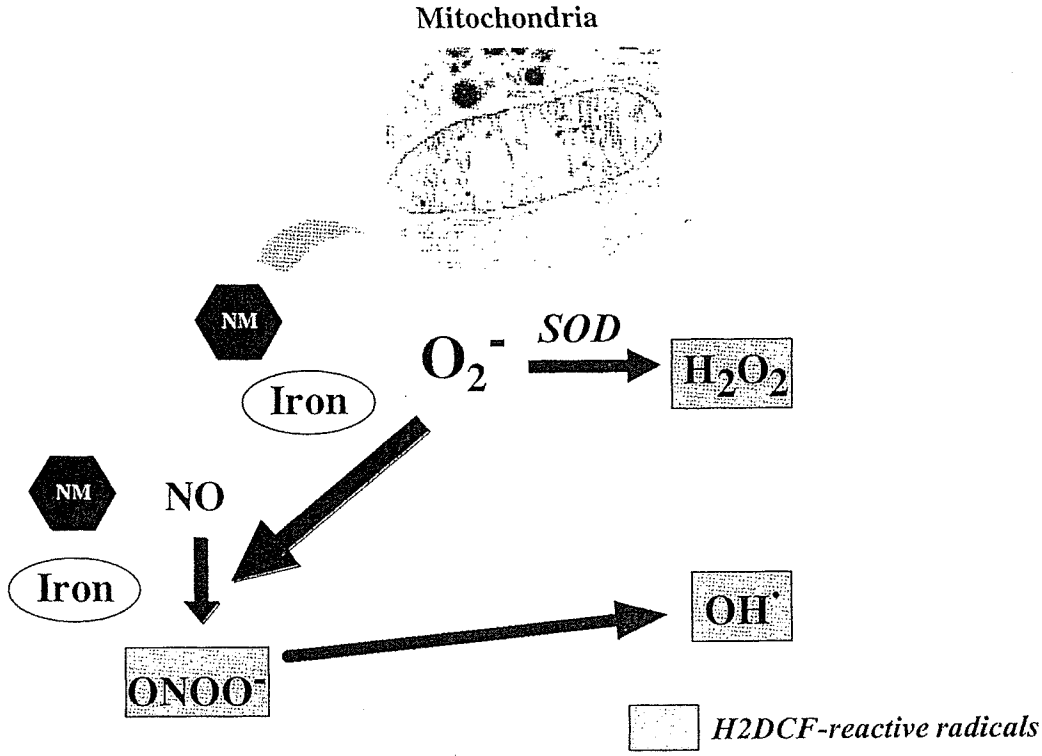

Fig. 5. The scheme how iron increases oxidative stress in mitochondria. Iron released from NM generates superoxide (O_2^-), which is catalized by SOD. O_2^- also reacts with nitric oxide (NO) by near-diffusion limit to produce peroxinitrite ($ONOO^-$). $ONOO^-$ is a strong radical itself and is decomposed into hydroxyl radical ($OH^\cdot$) also

reaction (Fig. 5). In the dopaminergic neurons, almost all the iron exists as bound with ferritin or NM, suggesting that NM may be neuroprotective, by chelating iron and other trace metals (Zecca et al., 2001b). Iron content in the substantia nigra increases by ageing but remained stable after the fourth decade of age, but NM content increases further according to ageing (Zecca et al., 2001a). In the postmortem PD brain, increased iron content was detected (Riederer et al., 1989) and recent results using transcranial ultrasonography revealed that in the substantia nigra of parkinsonian patients, not only the iron content increased, but that of NM decreased (Zecca et al., 2005). These results indicate that in the dopaminergic neurons in PD, the binding capacity of NM with iron was decreased and as a result, cytosolic free iron increased to inhibit ubiquitin-26S proteasome system, as shown in this paper (Gerlach et al., 2003).

Recent proteomics studies indicate the involvement of endosome-lysosome system as a source of proteinacious components in human NM (Tribl et al., 2005). This means NM production is not only "passive" accumulation of oxidative products, but some enzymatic system is required for the formation of high molecular aggregates. It requires further studies to elucidate whether the characteristics of NM itself or its synthetic pathway are changed in PD. The future studies on the intracellular mechanisms underlying the selective cell death by iron released from NM may bring out new strategies to prevent or rescue the decline in nigral dopamine neurons in PD.

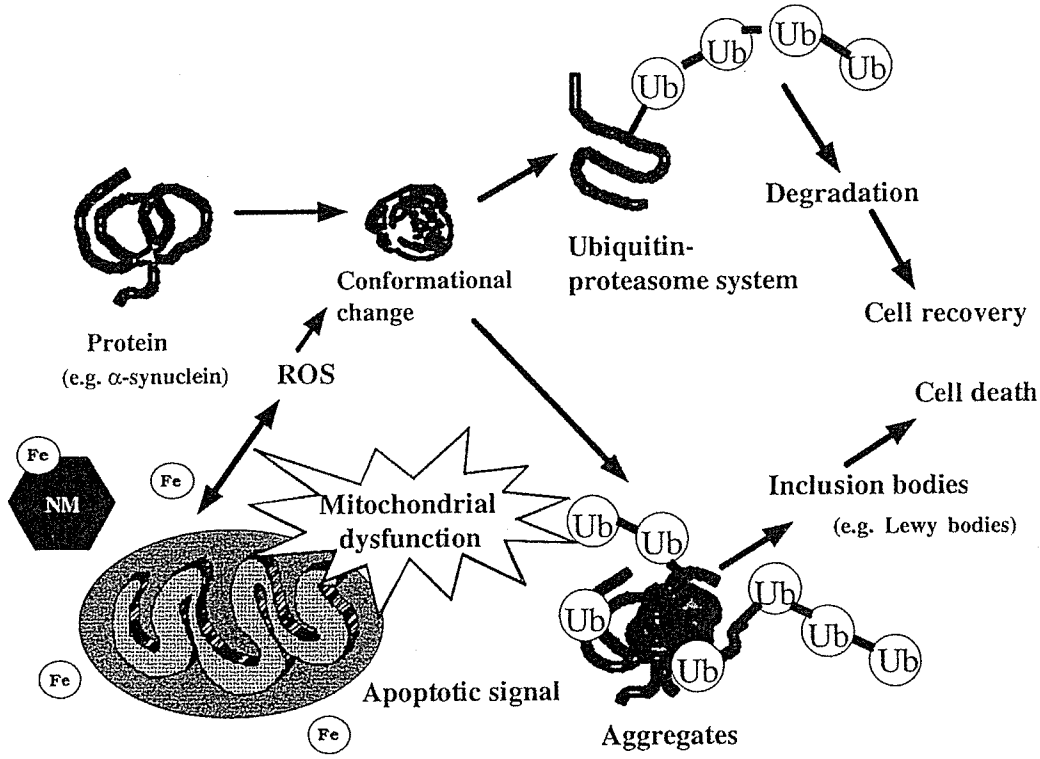

Fig. 6. The possible mechanism of dopaminergic neuronal death in Parkinson's disease. Iron released from NM increases oxidative stress in the mitochondria, and inhibit 26S proteasome system in the cells. Impairment of 26S proteasome increases accumulation of abnormal proteins to induce cell death

Acknowledgements

This work was supported by a Grant-in-Aid on Scientific Research for Young Scientists (B) for M. S. and (C) (W. M.) and (A) (W. M.) from Japan Society for the Promotion of Science, Grant for Research on Dementia and Bone Fracture (W. M., M. N.) from the Ministry of Health, Labor and Welfare, Japan.

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DOI 10.1007/s00702-005-0318-0
J Neural Transm (2006) 113: 125–135

Journal of Neural Transmission
Printed in Austria

Overexpression of amyloid precursor protein induces susceptibility to oxidative stress in human neuroblastoma SH-SY5Y cells

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Received October 12, 2004; accepted April 9, 2005
Published online June 15, 2005;

Summary. In Alzheimer's disease amyloid β peptide (Aβ) produced from amyloid precursor protein (APP) is considered to induce cell death. To clarify the molecular mechanism underlying Aβ neurotoxicity, we established the cell line overexpressing wild or mutant (His684Arg) APP in human SH-SY5Y cells. This paper presents that overexpression of wild-APP in the cells (SH/w-APP) increased the levels of APP and $A\beta_{1-40}$ but not $A\beta_{1-42}$, and reduced Bcl-2 level and proteasome activity with increased susceptibility to oxidative stress. The intracellular levels of reactive oxygen species in SH/w-APP increased significantly by H_2O_2 treatment. The level of Bcl-2 protein, but not mRNA, was markedly decreased in SH/w-APP cells, which was inversely correlated with APP expression among subcloned SH/w-APP cells. These results indicate that increased expression of wild type APP renders neuronal cells more vulnerable to oxidative stress leading to cell death.

Keywords: Alzheimer's disease, amyloid precursor protein, apoptosis, oxidative stress, proteasome inhibitor, Bcl-2.

Abbreviations

AD Alzheimer's disease; *AMC* 7-amino-4-methyl-coumarin; *APP* β-amyloid precursor protein; $A\beta_{1-40}$ β-amyloid$_{1-40}$; $A\beta_{1-42}$ β-amyloid$_{1-42}$; *CM-H_2DCFDA* 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate; *BOC* *t*-butyloxycarbonyl; *DCF* 2′,7′-dichlorofluorescein; *FACS* fluorescence augmented flow cytometry; *GDNF* glial cell line-derived neurotrophic factor; *GSH* reduced glutathione; *PBS* phosphate-buffered saline; *PD* Parkinson's disease; *PT* permeability transition; *RNS* reactive nitrogen species; *ROS* reactive oxygen species; *RT* reverse transcription; *Z* benzyloxycarbonyl.

Introduction

In Alzheimer's disease (AD), abnormal protein–protein interactions may induce precipitation of protein, forming intracellular neurofibrillary tangles composed of hyperphosphorylated tau, and extracellular aggregates of senile plaques containing β-amyloid (Aβ) (Selkoe and Schenk, 2003). The major constituent of the core of senile plaques is

Aβ, a 39–43 amino acid peptide, produced from β-amyloid precursor protein (APP) by a series of proteases, β- and γ-secretases. Under normal conditions, APP is processed by a non-amyloidogenic pathway, where α-secretase produces soluble form of APP, whereas by an amyloidogenic pathway β- and γ-secretases generate Aβ with 40 or 42 amino acids in the length, β-amyloid$_{1-40}$ or $_{1-42}$ (Aβ$_{1-40}$, Aβ$_{1-42}$). The involvement of Aβ$_{1-40}$ and Aβ$_{1-42}$ in AD pathogenesis has been shown further by studies on mutations in *APP* gene causing early-onset autosomal-dominant AD. *APP* mutations increase the ratio Aβ$_{1-42}$/Aβ$_{1-40}$ (Miravelle et al., 2000), and Aβ$_{1-42}$ is prone to precipitation and aggregation constituting amyloid fibrils. This suggests that abnormal processing of APP and increase in Aβ may play a major role in the pathogenesis of the hereditary forms and also that of sporadic AD, since the pathological features of both forms of AD are almost the same. In addition, genetic variability in Aβ catabolism and cleavage was reported to contribute to the risk of late-onset AD (Wavrant-DeVrieze et al., 1999). The "amyloid hypothesis" (Hardy and Selkoe, 2002) elucidates most of the sequential pathological processes in AD; Aβ$_{1-42}$ deposition in plaques, activation of microglia and astrocytes, and dysfunction of synapse and neurons.

Recently in AD, apoptosis has been implicated as the potentially important mechanism underlying neuronal cell loss (Cotman and Anderson, 1995; Stadelmann et al., 1999). Aβ and presenilins 1 and 2 were found to drive cells into apoptosis (Loo et al., 1993). A number of hypotheses have been proposed to elucidate the mechanisms behind the induction of apoptosis by Aβ in AD; direct toxicity, such as increased oxidative stress, mitochondrial dysfunction, perturbed ionic homeostasis, and altered activities of kinases and phosphatases (Mattson et al., 1992; Behl et al., 1994; Ghosh et al., 1999; Avila, 2000). At the same time, Aβ increases vulnerability of neuronal cells to various insults including oxidative stress (Goodman et al., 1994). More recently, new mechanistic aspects for the cell death in AD have been gathering attention. Aβ activates the mitochondrial apoptotic cascade, through opening permeability transition pore (PTP), which Bcl-2 protein family regulates, either in an anti-apoptotic way (Bcl-2, Bcl-xL) or in a pro-apoptotic function (Bax, Bad) (Zhang et al., 2002). In addition, Aβ induces dysfunction in the proteasome system, which is responsible for the cellular proteolysis of most ubiquitinated, oxidized protein and regulates oxidative stress (Keller et al., 2000).

In this paper, the effects of increased synthesis of APP, and Aβ$_{1-40}$ and Aβ$_{1-42}$ were studied in concern to the morphological characteristics of the cells, the susceptibility to oxidative stress and proteasome inhibition. For the present study we established human SH-SY5Y cells overexpressing the wild and the mutated APP at the 684th amino acid from His to Arg, which is within Aβ$_{1-40}$ and Aβ$_{1-42}$ region. The results are discussed in relation to the interaction among cellular factors, anti-oxidant capacities and mitochondrial Bcl-2 levels, the reduction of which may lower threshold to cell death.

Materials and methods

Chemicals

Peroxynitrate-generating 3-(4-morpholinyl)sydonimine (SIN-1) was purchased from Dojindo (Kumamoto, Japan); geneticin from Invitrogen (San Diego, CA, USA); a proteasome inhibitor, benzyloxycarbonyl (Z)-L-isoleucyl-γ-*t*-butyl-L-glutamyl-L-alanyl-L-leucinol (PSI), 7-amino-4-methyl-coumarin (AMC), substrates for proteasome, *t*-butyloxycarbonyl (Boc)-Leu-Arg-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Leu-Leu-Glu-MCA from Peptide Institute (Osaka, Japan). Hoechst 33342 and 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) were purchased from Molecular Probes (Eugene, OR, USA). Hydrogen peroxide (H_2O_2) and other chemicals were purchased from Wako (Tokyo, Japan). SH-SY5Y cells were cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan) supplemented with fetal calf serum in an atmosphere of 95% air-5% CO_2

at 37°C. Antibodies were purchased as follows: anti-β-tubulin antibody from Sigma (St. Louis, Mo, USA), rabbit polyclonal anti-APP from Cell Signaling (Beverly, MA, USA), and mouse monoclonal anti-Bcl2, anti-Bcl-xL, and rabbit polyclonal anti-Bax from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmid construction and DNA transfection of APP gene

Transfectants overexpressing wild APP were prepared with a pIRES1*neo* eukaryotic expression vector (Invitrogen, San Diego, CA, USA). For construction of pIRES1*neo-APP* vector, the coding region of human *APP* gene was obtained by reverse transcription (RT)-PCR from cDNA of SH-SY5Y cells and then inserted into the EcoRI-cleaved pIRES1*neo* vector. pIRES1*neo-APP* was confirmed to include wild-type *APP* gene by DNA sequencing. SH-SY5Y cells were transfected with pIRES1*neo* or pIRES1*neo-APP* by using liposomes (LipofectAMINE, GIBCO BRL, Rockville, MD, USA). The cells were selected using the culture medium containing 0.7 mg/ml geneticin (GIBCO BRL), and individual clones were isolated by limiting dilution, and characterized by RT-PCR and Western blot analysis. The stable clones overexpressing APP protein and control clone containing pIRES1*neo* vector were named as SH/w-APP and SH/IRES, respectively. The construction for an APP mutant, His684Arg, was performed by site-directed mutagenesis (BD Clontech, Franklin Lakes, NJ, USA) and the mutation was confirmed by DNA sequencing. The stable clone expressing APP mutant, His684Arg, was named as SH/m-684. In SH/w-APP cells, we further subcloned them by limiting dilution.

Quantitative real time RT-PCR

Total RNA was isolated using a Total RNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, USA). RNA samples were reverse transcribed by Super Script II RNase H-reverse transcriptase (Invitrogen) using oligo(dT) primer (Invitrogen). Prepared cDNA samples were purified by a PCR Purification kit (QIAGEN, Hilden, Germany) and used for PCR.

Quantitative real time PCR was performed using a LightCycler Fast Start DNA Master SYBR Green I kit (Roche, Mannheim, Germany) in a LightCycler (Roche) according to the manufacturer's instruction. Primers for *APP* were as follows: *APP* forward, 5′-AAGAAGGCAGTTATCCAGCATTTC-3′; for *APP* reverse, 5′-TTGTAGAGCAGGGAGAGAGACTGA-3′. Primers for *Bcl-2* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were commercially obtained from Roche. The PCR products were evaluated from the melting curve.

Quantitation of Aβ by the Sandwich ELISA

The culture medium of the cells cultured for 3 days was used for quantitative analyses of Aβ by the Sandwich ELISA for Aβ using Aβ ELISA kit (Biosource International, Camarillo, CA, USA). The values of Aβ concentrations in the medium were expressed as pg/ml. All ELISAs were carried out in duplicate.

Cell viability and morphological study

The vector-transfected SH-SY5Y cell clones grown in the presence of geneticin (G418, 0.7 mg/ml) were subjected to cell growth and morphological observation. Viable cell number was assessed by the trypan-blue dye exclusion test.

Immunocytochemistry

The cells were immunostained for β-tublin and F-actin as described in detail previously (Matsumoto et al., 2002). The cells were incubated with anti-β-tubulin antibody overnight at 4°C. After washing with PBS, the cells were incubated with the Alexa488-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. F-actin staining was performed with Alexa546-Phalloidin (Molecular Probes) for 30 min at room temperature. As negative control, we used the cells reacted with only the secondary antibody. The stained cells were observed with a fluorescence microscope, Olympus BX-51 (Olympus, Tokyo, Japan).

Detection of APP, Bcl-2, Bcl-xL and Bax by Western blotting analyses

Five micrograms of protein of each sample was analyzed for APP and Bcl-2 protein family, as previously described by Matsumoto et al. (2003). β-Tubulin was used as an internal control.

Measurements of production of reactive oxygen–nitrogen species by FACS

The production of reactive oxygen and nitrogen species (ROS-RNS) was quantitatively measured by the use of CM-H_2DCFDA, which detects H_2O_2, nitric oxide, and peroxynitrite. After treated with or without H_2O_2, the cells were washed twice with PBS, and CM-H_2DCFDA (10 μM in the final concentration) was added to the cell suspension in 25 mM HEPES, pH 7.5. After 30 min incubation at 37°C, the amount of 2′,7′-dichlorofluorescein (DCF) produced by CM-H_2DCFDA oxidation in the cell was determined by fluorescence-augmented flow cytometry (FACS) with a FACScaliber 4A and CellQuest software (Beckton Dickinson, San Jose, CA,

USA). The fluorometric measurement was performed with excitation at 488 nm and emission at 515–545 m.

Measurement of proteasome activity

The cells were suspended in the lysis buffer (50 mM Tris-HCl buffer, pH 7.75, 1 mM DTT, 0.5 mM EDTA and 5 mM $MgCl_2$) and incubated on ice for 30 min. The supernatants of centrifugation at 6000 g for 20 min were used as enzyme samples. Protein amount was measured with a DC Protein assay kit (Bio-Rad, Heoculos, CA, USA). Sample (10 μg protein) was incubated for 60 min at 37°C with MCA-conjugated substrates for three types of proteasome, Boc-Leu-

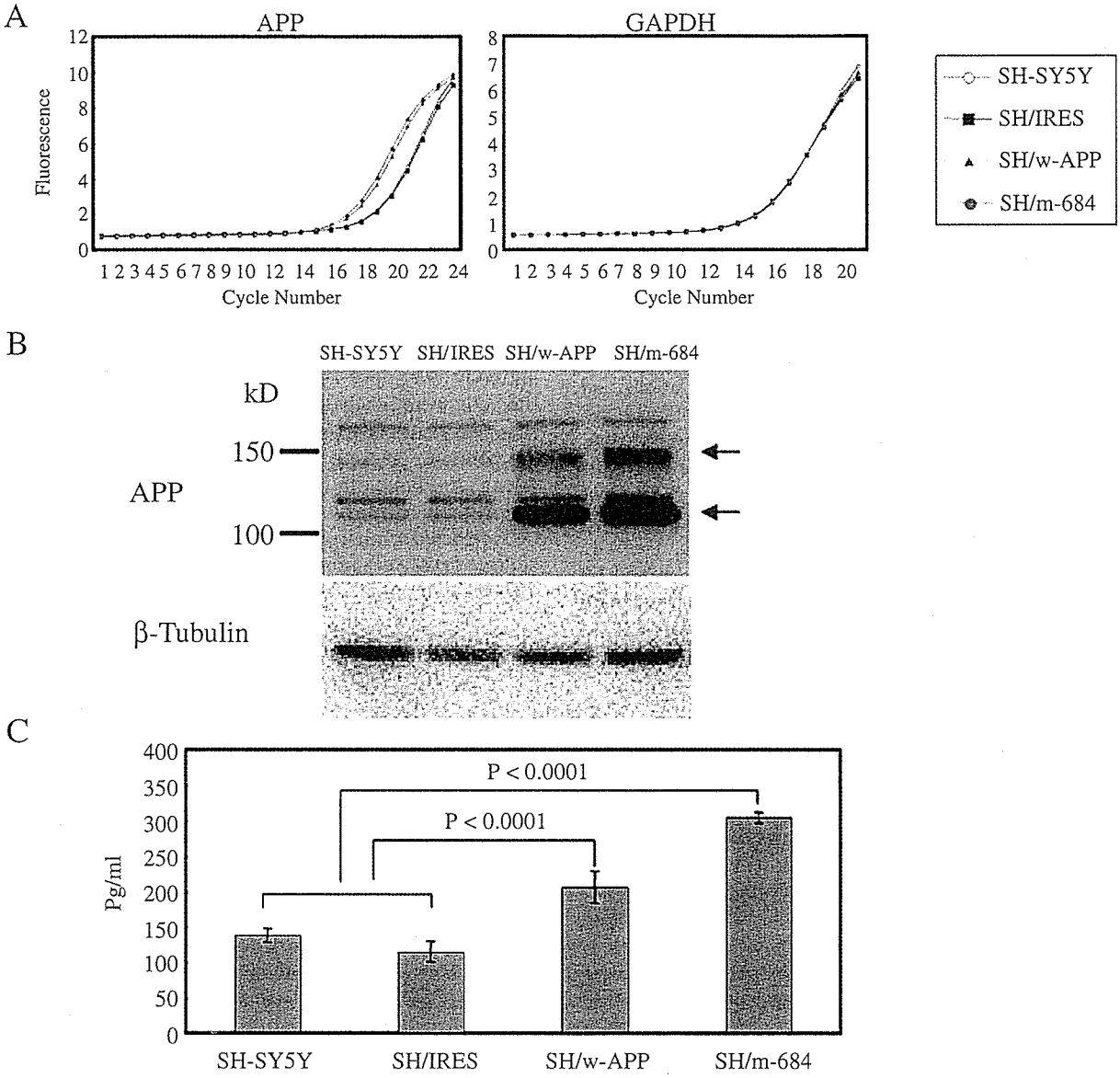

Fig. 1. Establishment of APP-overexpressing SH-SY5Y cells and assessment of the produced Aβ. **A** Real-time RT-PCR of *APP* and *GAPDH*. Cycle number and the fluorescent intensity corresponding to PCR product were shown. **B** Western blot analysis of APP. β-Tubulin was used as an internal standard. The arrows indicate the bands of APP. A band with the higher molecular weight corresponds to a modified APP probably by glycosylation and the lower one APP itself. **C** The amounts of $A\beta_{1-40}$ in the medium after 3 days' culture were assessed by ELISA. Difference from SH-SY5Y and SH/IRES is statistically significant

Arg-Arg-MCA for trypsin like, Suc-Leu-Leu-Val-Tyr-MCA for chymotrypsin like, and Z-Leu-Leu-Glu-MCA for postglutamyl peptidase-like activity. The released AMC fluorescence was measured in a fluoromicroplate reader, MTP-600 F (CORONA, Hitachinaka, Japan), with excitation at 380 nm and emission at 450 nm. The enzymatic activity was expressed as pmole/h/mg protein.

Measurement of intracellular GSH level

Intracellular reduced glutathione (GSH) content was quantitatively measured by using a GSH Assay Kit (Calbiochem, San Diego, CA). After cells were sonicated in 5% metaphosphoric acid and centrifuged at 3 000 g for 10 min, the supernatant was used to quantify GSH according to the manufacturer's instruction. The pellet dissolved in 1 M NaOH was used for the measurement of protein content. The GSH content was expressed as nmole/mg protein. Values were represented as the mean of three independent experiments.

Statistics

Differences were statistically evaluated by one way ANOVA followed by Fisher's PLSD. A p-value less than 0.05 was considered to be statistically significant.

Results

Establishment of APP-overexpressing SH-SY5Y cells

Real time RT-PCR study using *APP* specific P1 and P2 primers showed that the amounts of the products increased markedly in SH/w-APP transfected with wild APP, and SH/m-684 cells with mutated APP, compared to SH/IRES cells (Fig. 1A). The amount of APP protein of approximately 110 kDa and also modified form of APP with 140 kDa increased markedly in SH/w-APP and SH/m-684 cells, as determined by Western blot analysis (Fig. 1B). The amounts of $A\beta_{1-40}$ excreted in the culture medium are shown in Fig. 1C. $A\beta_{1-40}$ amounts significantly increased in SH/w-APP and SH/m-684 cells (Fig. 1C). The amount of $A\beta_{1-42}$ also increased in the culture medium of SH/m-684 cells to be 35 pg/ml, whereas it was under detection limit, 10 pg/ml, in that of other cell lines.

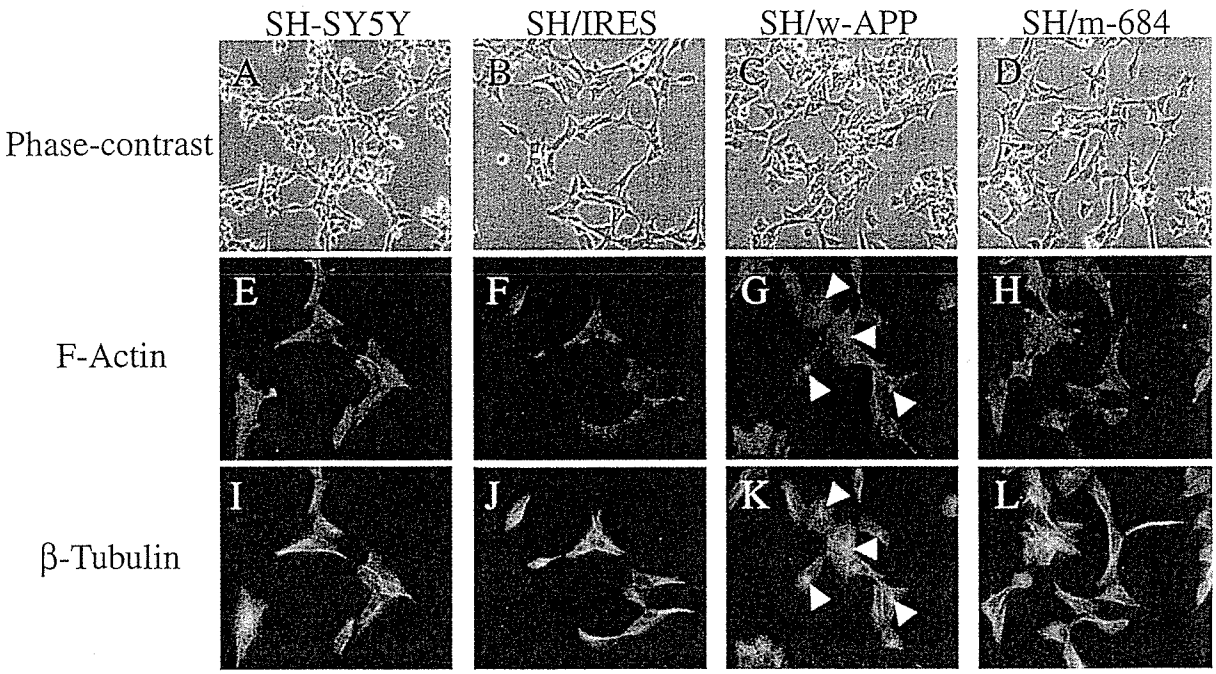

Fig. 2. Morphological study of the transfectants. *Upper panel*: phase-contrast aspects. *Middle and lower panel*: immunocytochemistry for F-Actin and β-Tubulin, respectively. The triangles indicate marked condensed regions of cytoskelton in SH/w-APP cells

Alteration of neurite net work formation in cells overexpressing wild-type APP

SH-SY5Y cells are usually sending out 1–2 long neurites, which contact with those of adjacent multiple cells forming a network with several cells. However, the morphological properties of SH/w-APP were markedly different from those of SH/IRES or SH/m-684 cells (Fig. 2A–D). SH/w-APP cells shaped polyhedral and had shorter and less branched neurites than other cell lines, which showed spindle form with neurites multi-directionally connected with adjacent cell bodies, as indicated in the staining of anti-F-actin and β-tubulin antibody (Fig. 2C).

Overexpression of wild APP sensitized the cells to oxidative stress, reduced proteasome activity, and increased GSH level

The effects of overexpression of wild and mutated APP on cell vulnerability were examined by exposing the cells to oxidative stress using H_2O_2, SIN-1 (a peroxynitrite donor), and PSI (an inhibitor of proteasome activity). The cells were treated with the agents for 12 h. The number of viable cells was shown in Fig. 3 and SH/w-APP was more sensitive to H_2O_2 than SH/IRES and SH/m-684. In addition, SIN-1and PSI also induced cell death more markedly in SH/w-APP than in other two cell lines.

The effects of APP overexpression on the ROS-RNS levels and the anti-oxidative capacity were examined by adding H_2O_2 to the culture medium. The intracellular ROS-RNS levels before and after H_2O_2 treatment were quantitatively determined as DCF produced from CM-H_2DCFDA (Fig. 4). The intracellular ROS-RNS levels of SH/w-APP cells significantly increased in a dose-dependent way (the middle panel), whereas in SH/IRES (the upper panel) and SH/m-684 cells (the lower panel) DCF levels were not affected by H_2O_2 (50 to 100 μM).

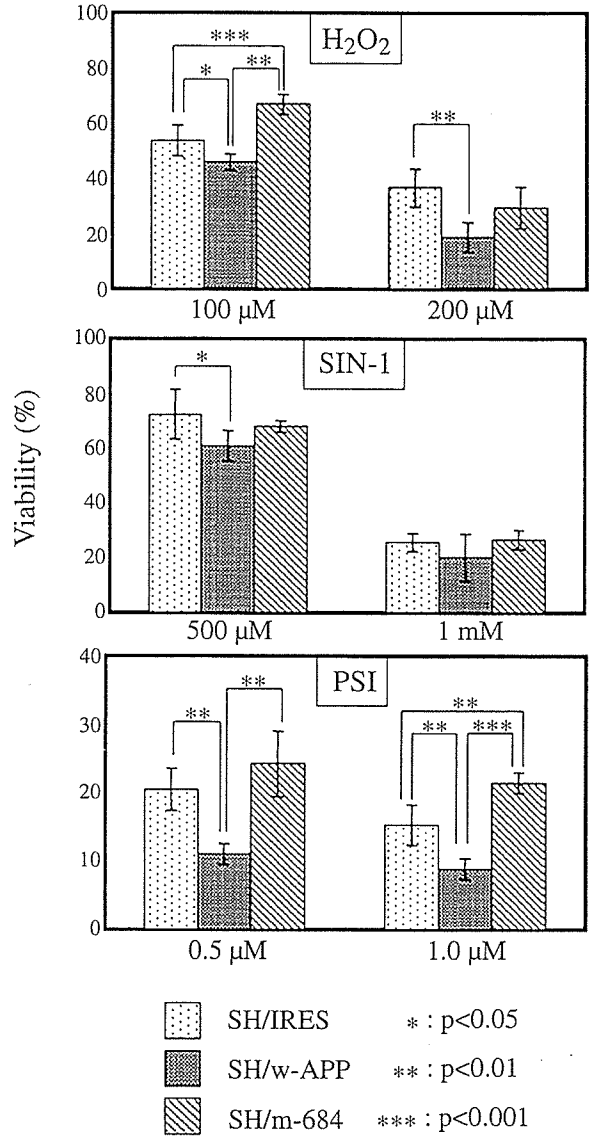

Fig. 3. The cell death induced y H_2O_2, SIN-1and PSI in the transfectants. Viable cell number was measured by trypan-blue dye exclusion test at 24 h after the treatment. *Upper panel*: Cells were treated with 100 and 200 μM H_2O_2. *Middle panel*: Treated with 500 μM and 1 mM SIN-1. *Lower panel*: Treated with 0.5 and 1 μM PSI. Data are expressed as means ± SD of four different experiments, and P values are evaluated by two-way ANOVA followed by Fisher's PLSD

The effect of APP overexpression on the activity of proteasome was examined in the cell lysate using synthetic fluorescent substrates without addition of ATP (Fig. 5). Trypsin-like activity of proteasome was significantly

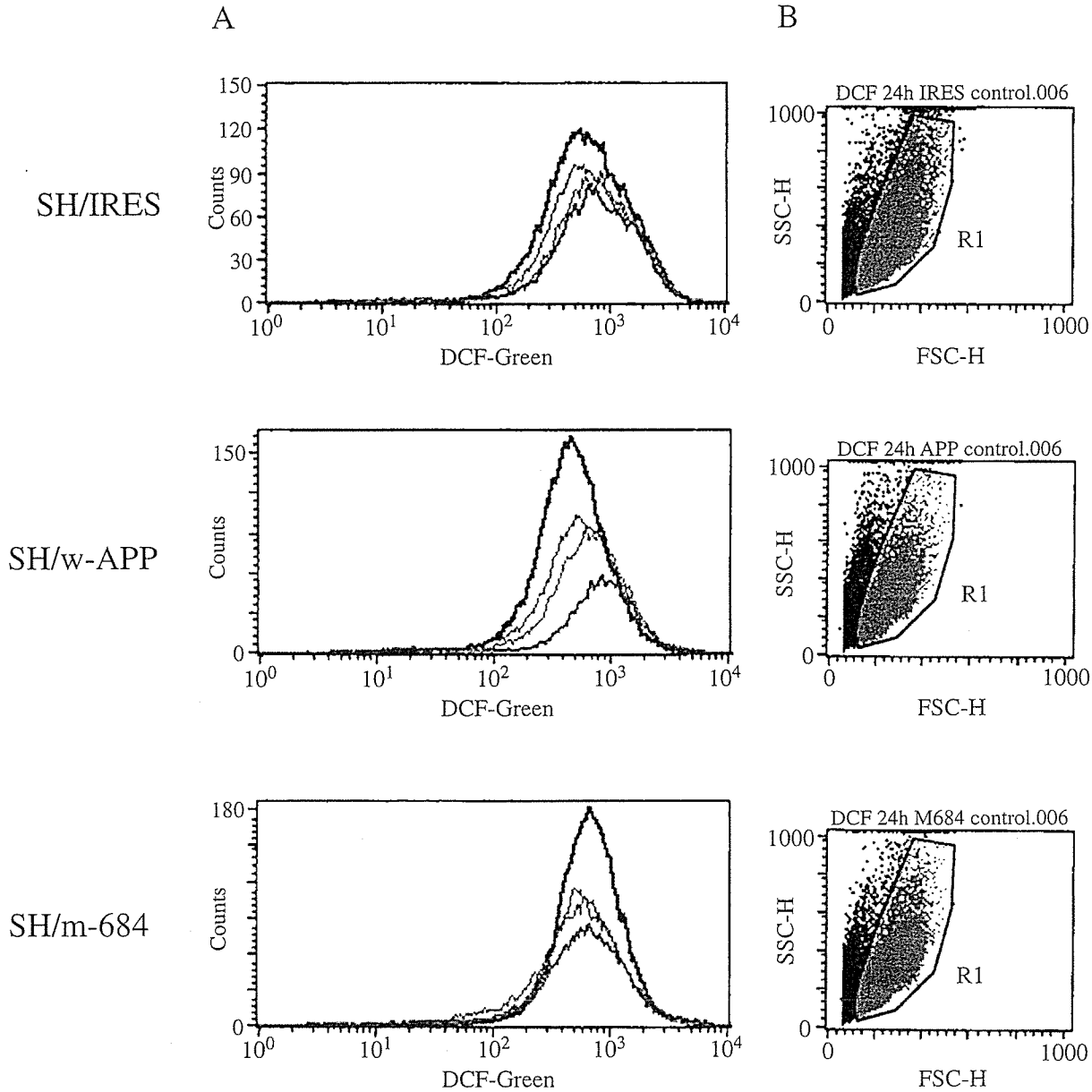

Fig. 4. The intracellular ROS-RNS levels after H_2O_2 treatment. The cells were treated with H_2O_2 for 24 h in serum-free medium. **A** The intracellular ROS-RNS levels measured by FACS using CM-H_2DCFDA. **B** Living cells were selected by FSC (forward scattered light)-H and SSC (side scattered light)-H (right panels)

reduced in SH/w-APP cells, whereas chymotrypsin-like and postglutamyl peptidase-like activities were only slightly reduced in SH/m-684 cells.

Moreover, as summarized in Table 1, the intracellular GSH levels were higher in SH/w-APP and SH/m-684 cells than control.

Effects of APP transfection on levels of Bcl-2 protein family

To clarify more detailed mechanism underlying oxidative stress-induced cell death, we examined the expression of Bcl-2 family protein regulating mitochondrial apoptotic mechanism in these transfectants. Among

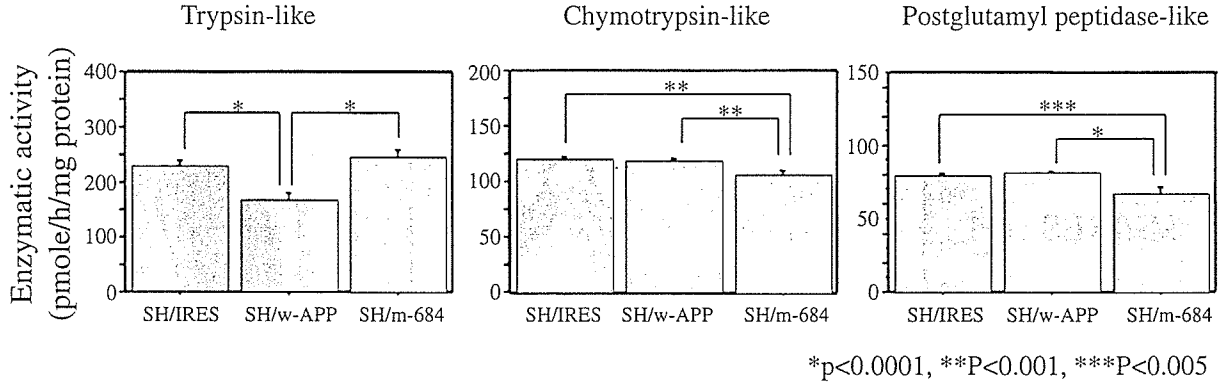

Fig. 5. The proteasome activity of the transfectants. The activities of trypsin-like, chymotrypsin-like and post-glutamyl peptidase-like proteasome were quantified by use of respective fluorescent substrates. The column and bar represent the mean and SD of four independent experiments. Difference is statistically significant

the Bcl-2 family tested, only the level of Bcl-2 protein was decreased exclusively in SH/w-APP cells, whereas it did not change in other transfectants (Fig. 6A). On the other hand, the levels of Bcl-xL and Bax were not affected by the transfection of wild and mutated APP vectors. However, the level of *bcl-2* mRNA measured by the quantitative real time RT-PCR was almost same among the transfectants (Fig. 6B). The effects of APP overexpression on the modification of Bcl-2 protein were examined in SH/w-APP cells, but we could not detect any changes in amounts of phosphorylated form of Bcl-2 protein. In the subcloned cells of SH/w-APP cells, the levels of APP were inversely correlated with those of Bcl-2, as shown in Fig. 6C.

Discussion

In this paper, it was studied whether the excess synthesis of wild-type APP/Aβ or of $A\beta_{1-42}$ or $A\beta_{1-40}$ is truly toxic and involved in neuronal cells. In SH/w-APP cells with increased wild type APP and $A\beta_{1-40}$, formation of synaptic connections was impaired. Previously, we exposed SH-SY5Y cells to synthetic $A\beta_{1-42}$, but did not detect any morphological change or cell death, suggesting that increased *in situ* synthesis of APP itself may be responsible for such morphological changes. This finding may be relevant with the fact that the similar changes were detected in brains at the early stage of AD (Arendt, 2001).

In the brains from patients with AD, increased oxidative stress has been confirmed by detection of carbonyls (Smith et al., 1996), nitrated protein (Good et al., 1996; Smith et al., 1997), and protein adducts with lipid peroxidation endproducts (Sayre et al., 1997). Oxidative stress has been considered either as causes or consequences of Aβ production (Butterfield and Lauderback, 2002), and recently Aβ was reported to induce mitochondrial dysfunction through the conformational change in binding site with nicotinamide adenine dinucleotide, which may increase

Table 1. Intracellular levels of reduced glutathione in the transfectants

Transfectants	SH/IRES	SH/w-APP	SH/m-684
GSH content (nmole/mg protein)	20.05 ± 1.62	28.30 ± 1.73*	27.30 ± 1.56*

The number represents mean ± SD of 6 experiments. * $p < 0.01$ from SH/IRES

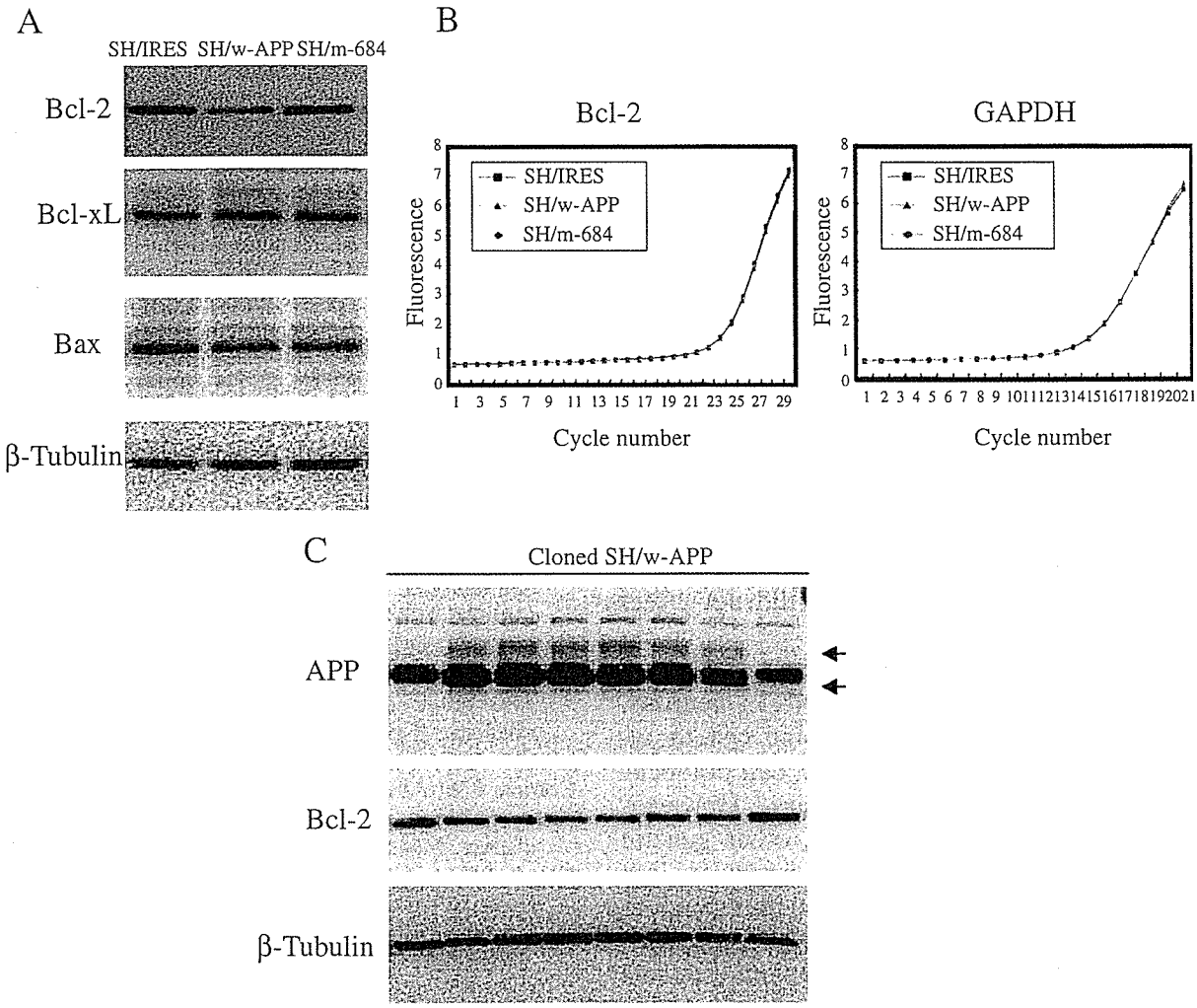

Fig. 6. The expression of apoptosis-related Bcl-2 family in the transfectants and the association of the expression between APP and Bcl-2 expression in SH/w-APP cells. **A** Western blot analysis of Bcl-2, Bcl-xL and Bax and β-tubulin used as an internal standard, in SH/IRES, SH/w-APP and SH/m-684 cells. **B** Real-time RT-PCR of *Bcl-2* and *GAPDH*. Cycle number and the fluorescent intensity corresponding to PCR product were shown. **C** Western blot analysis of APP, Bcl-2 and β-tubulin in subcloned cells overexpressing APP

oxidative stress (Lusbader et al., 2004). SH/w-APP cells overexpressing APP could survive and keep proliferation, and the level of ROS-RNS was not increased in steady state, even though anti-oxidative capacity was impaired, since H_2O_2 loading increased ROS-RNS and reduced the activity of proteasome, "the second scavenger" of ROS-RNS. Exposure to H_2O_2 or a proteasome inhibitor turned the compensating state in SH/w-APP cells into un-compensating state, further increased oxidative stress, and cell death was induced more markedly than in other transfectants.

The levels of anti-apoptotic Bcl-2 protein were reduced only in SH/w-APP, and the reduced levels were in an inverse correlation with the increased APP levels, which may be relevant with a previous report that Aβ peptides down-regulated Bcl-2 and upregulated Bax expression (Paradis et al., 1996). Selective reduction of Bcl-2 protein in SH/w-APP may be relevant with the increased sensitivity

to oxidative stress in this cell line. The ability of Bcl-2 to inhibit apoptosis induced by Aβ was reported to depend on its expression level (Ivins et al., 1999), supporting further our view. Anti-apoptotic Bcl-2 at sufficient concentrations regulates mitochondrial PT pore, and prevents apoptosis (Maruyama et al., 2002; Akao et al., 2003). It should be emphasized that not the mRNA level but the protein level of Bcl-2 was decreased in SH/w-APP cells. Since the phosphorylated Bcl-2 was not changed in our case, another posttranslational modification(s) may be involved in reduction of Bcl-2 protein. As a conclusion, only overexpression of wild type APP altered functional and morphological properties in the cells, while expression of mutated Aβ increased $A\beta_{1-42}$ excretion, but did not affect cell properties.

Mutations in APP, presenilin 1 and 2, and ApoE4 may increase ROS-RNS in neurons, but for a while the anti-oxidant system including the proteasome system can compensate enhanced oxidative stress. However, further insults, such as aging, other genetic or environmental factors, reduce anti-oxidant capacity further and lower the threshold to apoptosis. If we can control the increased oxidative stress and cell death using anti-oxidants (Gilgun-Sherki et al., 2003), or so-called neuroprotective agents, such as rasagiline, which induce the expression of pro-survival genes coding Bcl-2, Bcl-xL and neurotrophic factors (Akao et al., 2002; Maruyama et al., 2003, 2004; Weinstock et al., 2000), there will be a chance to protect neurons from cell death in AD. More work will be required for dissolving this problem.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (Y. A., No. 09670859), on Scientific Research (C) (W. M.) and Center of Excellence Research Grant (Y. A.) from the Ministry of Education, Culture, Sports and Science and Technology, Japan, Grant-in-Aid for Scientific Research on Priority Areas (C) from Japan Society for the Promotion of Science (W. M.), Grant for Dementia and Bone Fracture (W. M., and M. N.) from the Ministry of Health, Labor and Welfare, Japan.

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