Discussion

The clinical trials to prove the neuroprotective function of rasagiline and (−)deprenyl were reported, but the results are still contradicting, and biomarkers to estimate the progression of neuronal loss should be invented (Michell et al., 2004). The markers for the disease progression and treatment efficiency are based on clinical evaluation of symptoms, PET and SPECT imaging, transcranial ultrasound and some biochemical tests. However, blood tests for PD progression are limited to monitor the pathogenic factors, such as increased oxidative stress (malondialdehyde, superoxide radicals, 8-hydrox-2′-deoxyguanosine), or the reduced complex I (Schapira et al., 1990) and increased MAO-B activity in platelets (Zhou et al., 2001). (−)Deprenyl may reverse the increase in MAO-B and the subsequent reduction of β-phenylethylamine in plasma, but these markers represent MAO inhibitory function of (−)deprenyl, but not the neuroprotective activity. α-Synuclein and its phosphorylated proteins were proposed as the markers, but the recent results did not support this view. In CSF, increased levels of 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-guanosine and malondialdehyde were detected (Abe et al., 2003). However, these markers do not present information for progression of selective neuronal loss in PD.

At present, mechanistic makers for factors intervening the disease progress may be the only available markers to assess the neuroprotective potency. As described above, rasagiline induces GDNF in cultured cells, suggesting that the levels of neurotrophic factors specific for dopamine neurons may be used as markers. Indeed, we examined the change in neurotrophic factors in monkey CSF after systemic treatment of rasagiline (Maruyama et al., in preparation). The results proved the validity of our view, which was supported further by the analyses of the CSF from Parkinsonian patients before and after treatment of (−)deprenyl, even the limited number of the samples (Maruyama et al., in preparation). We are now examining the candidates of the biomarkers for the neuroprotective function in serum and CSF from Parkinsonian patients and primate models.

Recently, an increasing number of evidences indicate that rasagiline and related compounds can ameliorate pathogenic processes in AD and other neurodegenerative disorders. Rasagiline analogues with inhibitor potency to cholinesterase, TV 3326, and its S enantiomer TV 3279 were reported to regulate the processing of amyloid precursor protein (APP) and increase the soluble APP secretion through activation of α-secretase activity and the reduction of holo-APP protein (Youdim et al., 2003b;

Yogev-Falach et al., 2006). Their results suggest that propargylamines intervene the pathogenic processes in neurodegenerative disorders in general and ameliorate the disease process.

The stereo-chemical and enantiomeric specificity of the propargylamine for their neuroprotective activity suggests the occurrence of the target protein in mitochondria and other cell components. The identification of the binding site of neuroprotective propargylamines may give us a clue to find the most adequate chemical structure for the function, and develop new drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

Acknowledgement

References

Abe T, Isobe C Murata T, Sato C, Tohgi H (2003) Alteration of 8-hydroxyguanosine concentrations in the cerebrospinal fluid and serum from patients with Parkinson's disease. Neurosci Lett 336: 105–108

Akao Y, Maruyama W, Shimizu S, Yi H, Nakagawa Y, Shamoto-Nagai M, Youdim MBH, Tsujimoto Y, Naoi M (2002a) Mitochondrial permeability transition mediates apoptosis induced by N-methyl(R)salsolinol, an endogenous neurotoxin, and is inhibited by Bcl-2 and rasagiline, N-propargyl-1(R)-aminoindan. J Neurochem 82: 913–923

Akao Y, Maruyama W, Yi H, Shamoto-Nagai M, Youdim MBH, Naoi M (2002b) An anti-Parkinson's disease drug, N-propargyl-1(R)-aminoindan (rasagiline), enhances expression of anti-apoptotic Bcl-2 in dopaminergic SH-SY5Y cells. Neurosci Lett 326: 105–108

Bauer M, Meyer M, Grimm L, Meitinger T, Zimmer J, Gasser T, Ueffing M, Widmer HR (2000) Nonviral glial cell-derived neurotrophic factor gene transfer enhances survival of cultured dopaminergic neurons and improves their function after transplantation in a rat model of Parkinson's disease. Hum Gene Ther 11: 1529–1541

Birkmayer W, Riederer P, Ambrozi L, Youdim MBH (1977) Implication of combined treatment with "Madopar" and L-deprenyl in Parkinson's disease. A long-term study. Lancet 309: 439–443

Brabeck C, Pfeiffer R, Leake A, Beneke S, Meyer R, Bürkle A (2003) L-Selegiline potentiates the cellular poly(ADP-ribosyl)ation response to ionizing radiation. J Pharm Exp Ther 306: 973–979

Carrillo MC, Minami C, Kitani K, Maruyama W, Ohashi K, Yamamoto T, Naoi M, Kanai K, Youdim MBH (2000) Enhancing effect of rasagiline on superoxide dismutase and catalase activities in the dopaminergic system in rat. Life Sci 67: 577–585

Dawson TM, Dawson VL (2002) Neuroprotective and neurorestorative strategies for Parkinson's disease. Nature Neurosci Suppl 5: 1058–1061

Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou J-C (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 144: 891–901

Eckert A, Steiner B, Marques C, Leutz S, Roming H, Haass C, Muller WE (2001) Elevated vulnerability to oxidative tress-induced cell death and activation of caspase-3 by the Swedish amyloid precursor protein mutation. J Neurosci Res 64: 183–192

Eriksen JL, Wszolek Z, Petrucelli L (2005) Molecular pathogenesis of Parkinson disease. Arch Neurol 62: 353–357

Everett H, Barry M, Sun X, Lee SF, Franz C, Berthiaume LG, McFadden G, Bleackley RC (2002) The myxoma provirus protein, M11L, prevents apoptosis by direct interaction with the mitochondrial permeability transition pore. J Exp Med 196: 1127–1139

Gill SS, Patel N, Hotton CR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 9: 589–595

Hara RH, Thomas B, Cascio MB, Bae B-I, Hester LG, Dawson VL, Dawson TM, Sawa A, Synder SH (2006) Neuroprotection by pharmacological blockade of the GAPDH death cascade. Proc Natl Acad Sci USA 103: 3887–3889

Hirai C, Takahata K, Shimizu S, Yoneda F, Hayashi K, Katuki H, Akaike A (2005) Effects of R-(−)-BPAP on the expressions of neurotrophins and their receptors in mesencephalic slices. Biol Pharm Bull 28: 1524–1526

Jacotot E, Ferri KF, Hamel CEl, Brenner C, Druillennec S, Hoebeke J, Rustin P, Metivier D, Lenoir C, Geuskens M, Vierira HLA, Loeffler M, Belzaq A-S, Briand J-P, Zamzami N, Edelman L, Xie ZH, Reed JC, Roques N, Kroemer G (2001) Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV viral protein R and Bcl-2. J Exp Med 193: 509–519

Kitani K, Minami C, Maruyama W, Kanai S, Ivy GO, Carrillo MC (2000) Common properties for propargylamines of enhancing superoxide dismutase and catalase activities in the dopaminergic system in the rat: implications for the life prolonging effect of (−)deprenyl. J Neural Transm Suppl 60: 139–156

Kroemer G (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 3: 614–620

Lucken-Ardjomande S, Martinou J-C (2005) Newcomers in the process of mitochondrial permeabilization. J Cell Sci 118: 473–483

Mandel S, Grünblatt E, Riederer P, Gerlach M, Levites Y, Youdim MBH (2003) Neuroprotective strategies in Parkinson's disease. CNS Drugs 17: 729–762

Mandel S, Weinreb O, Amit T, Youdim MBH (2005) Mechanism of neuroprotective action of the anti-Parkinson drug rasagiline and its derivatives. Brain Res Rev 48: 379–387

Maruyama W, Bouton AA, Davis BS, Dostert P, Naoi M (2001a) Enantio-specific induction of apoptosis by an endogenous neurotoxin, N-methyl(R)salsolinol, in dopaminergic SH-SY5Y cells: suppression of apoptosis by N-(2-heptyl)-N-methylpropargylamine. J Neural Transm 108: 11–24

Maruyama W, Youdim MBH, Naoi M (2001b) Antiapoptotic properties of rasagiline, N-propargylamine-1(R)-aminoindan, and its optical (S)-isomer, TV1022. Ann NY Acad Sci 939: 320–329

Maruyama W, Akao Y, Youdim MBH, Davis BA, Naoi M (2002a) Transfection-enforced Bcl-2 overexpression and an anti-Parkinson drug, rasagiline, prevent nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase induced by an endogenous dopaminergic neurotoxin, N-methyl(R)salsolinol. J Neurochem 78: 727–735

Maruyama W, Akao Y, Carrillo MC, Kitani K, Youdim MBH, Naoi M (2002b) Neuroprotection by propargylamines in Parkinson's disease. Suppression of apoptosis and induction of prosurvival genes. Neurotoxicol Tertol 24: 675–682

Maruyama W, Takahashi T, Youdim MBH, Naoi M (2002c) The anti-parkinson drug, rasagiline, prevents apoptotic DNA damage induced by peroxynitrite in human dopaminergic neuroblastoma SH-SY5Y cells. J Neural Transm 109: 467–481

Maruyama W, Winstock M, Youdim MBH, Nagai M, Naoi M (2003) Anti-apoptotic action of anti-Alzheimer drug, TV3326 [(N-propargyl)-(3R)-aminoindan-5-yl]-ethyl methyl carbamate, a novel cholinesterase-monoamine oxidase inhibitor. Neurosci Lett 341: 233–236

Maruyama W, Nitta A, Shamoto-Nagai M, Hirata H, Akao Y, Furukawa S, Nabeshima T, Naoi M (2004a) N-Propargyl-1(R)-aminoindan, rasagiline, increases glial cell line-derived neurotrophic factor (GDNF) in neuroblastoma SH-SY5Y cells through activation of NF-κB transcription factor. Neurochem Int 44: 393–400

Maruyama W, Yi H, Takahashi T, Shimizu S, Ohde H, Yoneda F, Iwasa K, Naoi M (2004b) Neuroprotective function of R-(−)-(benzofuran-2-yl)-2-propylamino-pentane, [R-(−)-BPAP], against apoptosis induced by N-methyl(R)salsolinol, an endogenous dopaminergic neurotoxin, in human dopaminergic neuroblastoma SH-SY5Y cells. Life Sci 75: 107–117

McStay GP, Clarke SJ, Halestrap AP (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem J 367: 541–548

Michell AW, Lewis SJG, Barker RA (2004) Biomarkers and Parkinson's disease. Brain 127: 1693–1705

Naoi M, Maruyama W, Akao Y, Yi H (2002a) Dopamine-derived endogenous N-methyl-(R)-salsolinol. Its role in Parkinson's disease. Neurotoxicol Teratol 24: 579–591

Naoi M, Maruyama W, Akao Y, Yi H (2002b) Mitochondrial determine the survival and death in apoptosis by an endogenous neurotoxin, N-methyl(R)salsolinol, and neuroprotection by propargylamines. J Neural Transm 109: 607–621

Naoi M, Maruyama W, Akao Y, Yi H, Yamaoka Y (2006) Involvement of type A monoamine oxidase in neurodegeneration: regulation of mitochondrial signaling leading to cell death or neuroprotection. J Neural Transm Suppl 71: 67–77

Narita M, Shimizu S, Ito T, Chittenden T, Litz RJ, Matsuda H, Tsujimoto Y (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci USA 95: 14681–14686

Nitta A, Murai R, Maruyama K, Furukawa S (2002) FK506 protects dopaminergic degeneration through induction of GDNF in rodent brains. In: Mizuno Y, Fisher A, Hanin I (eds) Mapping the progress of Alzheimer's and Parkinson's disease. Kluwer Academic/Plenum Publishers, New York, pp 446–467

Pälhagen S, Heinonen E, Hägglund J, Kaugesaar T, Mäki-Ikola O, Palm R, the Swedish Parkinson Study Group (2006) Selegiline slows the progression of the symptoms of Parkinson disease. Neurology 66: 1200–1206

Parkinson Study Group (2002) A controlled trial of rasagiline in early Parkinson disease. Arch Neurol 59: 1937–1943

Parkinson Study Group (2004) A controlled, randomized, delayed-start study of rasagiline in early Parkinson disease. Arch Neurol 61: 561–566

Riederer P, Lachenmayer L, Laux G (2004) Clinical applications of MAO-inhibitors. Curr Med Chem 11: 2033–2043

Riederer PF (2004) Views on neurodegeneration as a basis for neuroprotective strategies. Med Sci Monit 10: RA287–290

Sagi Y, Mandel S, Amit T, Youdim MBH (2007) Activation of tyrosine kinase receptor signaling pathway by rasagiline facilitates neurorescue and restoration of nigrostriatal dopamine neurons in post-MPTP-induced parkinsonism. Neurobiol Dis 25: 35–44

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54: 823–827

Schapira AHV, Olanow CW (2004) Neuroprotection in Parkinson disease. Mysteries, myths and misconceptions. JAMA 291: 358–364

Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, Matsuda H, Tsujimoto Y (1998) Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc Natl Acad Sci USA 95: 1455–1459

Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399: 483–487

Simpkins N, Jankovic J (2003) Neuroprotection in Parkinson disease. Arch Intern Med 163: 1650–1654

Suchowersky O, Gronseth G, Permutter J, Reich S, Zesiewics T, Weiner WJ (2006) Practice parameter: neuroprotective strategies and alternative therapies for Parkinson disease (an evidence-based review). Neurology 66: 976–982

Tatton WG, Chalmers-Redman RM, Ju WJ, Mammen M, Carlile GW, Pong AW, Tatton NA (2002) Propargylamines induce antiapoptotic new protein synthesis in serum- and nerve growth factor (NGF)-withdrawn, NGF-differentiated PC-12 cells. J Pharmacol Exp Ther 301: 753–764

Tsujimoto Y, Shimizu S (2000) Bcl-2 family: Life-or-death switch. FEBS Lett 466: 6–10

Vila M, Przedborski S (2004) Genetic clues to the pathogenesis of Parkinson's disease. Nat Med 10: S58–S62

Weinreb O, Bar-Am O, Amit T, Chillag-Talmor O, Youdim MBH (2004) Neuroprotection via pro-survival protein kinase C isoforms associated with Bcl-2 family protein. FASEB J 18: 1471–1473

Yi H, Akao Y, Maruyama W, Chen K, Shih J, Naoi M (2006a) Type A monoamine oxidase is the target of an endogenous dopaminergic neurotoxin, N-methyl-(R)salsolinol, leading to apoptosis in SH-SY5Y cells. J Neurochem 96: 541–549

Yi H, Maruyama W, Akao Y, Takahashi T, Iwasa K, Youdim MBH, Naoi M (2006b) N-Propargylamine protects SH-SY5Y cells from apoptosis induced by an endogenous neurotoxin, N-methyl(R)salsolinol, through stabilization of mitochondrial membrane and induction of anti-apoptotic Bcl-2. J Neural Transm 113: 21–32

Yogev-Falach M, Bar-Am O, Amit T, Weinreb O, Youdim MBH (2006) A multifunctional, neuroprotective drug, ladostigil (TV3326), regulates holo-APP translation and processing. FASEB 20: 2177–2179

Youdim MBH, Gross A, Finberg JPM (2001) Rasagiline [N-propargyl-1R(+)-aminoindan], a selective and potent inhibitor of mitochondrial monoamine oxidase B. Br J Pharmacol 132: 500–506

Youdim MBH, Amit T, Falach-Yogev M, Am OB, Maruyama W, Naoi M (2003a) The Essentiality of bcl-2, PKC and proteasome-ubiquitin complex activations in the neuroprotective-antiapoptotic action of the anti-Parkinson drug, rasagiline. Biochem Pharmacol 66: 1635–1641

Youdim MB, Amit T, Bar-Am O, Weinstock M, Yogev-Falach M (2003b) Amyloid processing and signal transduction properties of antiparkinson-antialzheimer neuroprotective drugs rasagiline and TV3326. Ann NY Acad Sci 993: 378–386

Youdim MBH, Edmondson D, Tipton KF (2006) The therapeutic potential of monoamine oxidase inhibitors. Nature Rev Neurosci 7: 295–309

Zhou G, Miura Y, Shoji H, Yamada S, Matsushi T (2001) Platelet monoamine oxidase B and plasma beta-phenethylamine in Parkinson's disease. J Neurol Neurosurg Psychaitry 70: 229–231

Oxidation of polyunsaturated fatty acids induces protein oligomerization and may initiate neuronal death process in Parkinson's disease

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INTRODUCTION

Long chain polyunsaturated fatty acids (PUFA), are enriched in the nervous system. Docosahexaenoic acid (22:6n-3, DHA) is one of the most abundant PUFA in the brain and retina, and is component of phosphatidylethanolamine and phosphatidylserine in the celluler membrane. Administration of DHA to the rodents improves their brain functions (1) and it is suggested DHA in the membrane plays a role in the synaptic plasticity and signal transduction systems in the neuronal cells (2-4). In the human, lipids orally administrated are reconstructed through α-oxidation and elongation reaction mainly in the liver after digestion and absorption. However, human can not synthesize enough amount of DHA, so, the intake of DHA from food, mainly fish oil, is essentially required (essential fatty acid). Brain DHA is derived from the circulating plasma pool across the Blood-Brain-Barrier. In the human brain, the amount of n-6 PUFA, such as DHA is estimated to be 17.1% of the total fatty acids, and on the other hand, the amount of n-3 PUFA, such as arachidonic acid (AA) is estimated to be 9.7%. The concentration of n-3 PUFA is higher than that of plasma, so that, the existence of specific transporter for DHA is suggested in the neural cells and astrocytes (5). DHA is known to be a potent antioxidant, but

simultaneously, it is easily oxidized and produces toxic lipid peroxide. These toxic lipid peroxide and their products, such as aldehyde may form adduct with the proteins to induce degenerated proteins with abnormal conformation. The proteins, which associated with cell membrane, such as cell-surface receptor, membrane anchoring protein, or functionally transmembrane protein are the candidates. The oxidized products are degradated by ubiquitin-proteasome system or autophagy, but according to ageing, the accumulation of these abnormal proteins might perturb homeostasis to induce neuronal death.Parkinson disease (PD) is the second common neurodegenerative disorder and affects 1-2% of aged population over 60 years old. The pathogenesis of PD has not been clarified, but aggregation of protein with abnormal conformational is commonly observed features in the neurodegenerative disorders. In PD, degeneration of dopamine neuron in the substantia nigra and the existence of Lewy boies (LB), are diagnostic pathological features. α-Synuclein (α-Syn) is the main component of LB and is known to exist as membrane-bound form by association with PUFA. The mutation of A53T, A30P, and E46K, or triplication of α-Syn gene have been identified in early-onset familial PD (6-8). It is suggested that accumulation of α-Syn is the cause, not the result of PD. In sporadic PD without α-Syn mutation, some post-translational modification of α-Syn may induce pathological process similar to familial PD. α-Syn is a 140-amino acid protein expressed ubiquitously in the neuron and accounts for 0.1-1% of brain cytosolic proteins. α-Syn is suggested to play many roles in nervous system, including regulation of synaptic vesicle mobilization, chaperone activity, modulation of dopamine transporter and dopamine biosynthesis (9-11). In this paper, the oxidative modification of α-Syn by lipid peroxide and aldehyde derived from PUFA was examined. The possibility that lipid peroxidation in the membrane-composing PUFA and adduct formation with α-Syn was investigated. The results are discussed in relation to the role of oxidative stress in ageing in the pathogenesis of PD.

MATERIALS AND METHODS

PUFA is oxidized and produce lipid peroxides, then, form adducts with lysine residue in the proteins. DHA is oxidized and split to produces succinyl-lysine adduct (SUL) with carbonyl terminal and propanoyl-lysine adduct (PRL) with amino terminal, respectively. AA is also oxidized, to produce glutaroyl-lysine (GLL) with carbonyl terminal and hexanoyl-lysine (HEL) adduct with amino terminal. The antibodies of these 4 oxidized PUFA products, SUL, PRL, GLL and HEL are prepared as reported previously (12). Recombinant α-Syn is purachased from BIOMOL International. L.P. (Plymouth Meeting, PA.). DHA, AA and oleic acid donated from CAYMAN CHEMICAL (Ann Arbor, MI) and stearic acid are Nu-chek prep, Inc. (Elysian, MN). Other chemicals are from WAKO finechemical.(Osaka, Japan).

Recombinant α-Syn (2 μM) was co-incubated with long chain fatty acids namaly DHA, AA, oleic acid or stearic acid (1-10 mM) for 3 to 7 days. The reaction products were separated by SDS-PAGE and were analyzed by western blotting using anti-α-Syn antibody. The sample incubated

with DHA or AA, the production of oxidized PUFA was examined also, using antibodies described above. The effects of antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), on oligomerization of α-Syn was examined also.

We examined whether DHA is neuroprotective or neurotoxic using human clonal neuralblastoma SH-SY5Y cells. Free DHA (0-100 μM) or DHA (0-100 μM) pre-incubated with 4 folds concentration of Bovine serum albumin (BSA), which acts as lipid carrier protein was added to the culture medium in the presence or absence of tocopherol (100 μM) for 2 days in 5% CO_2-95% atmosphere at 37 °C. Culture medium was COS-Medium 001 (COSMOBIO, Japan) without serum.. Cell death was estimated using LDH assay according to manufacture's procedure. The cells were mechanically harvested and gathered, then homogenized in RIPA Buffer, then centrifuged at 10,000 xg for 15 min. The supernatant and pellet were used as soluble and insoluble fraction, respectively. The amount of lipid peroxides in the soluble and insoluble fraction of the cells was estimated by fluorospectmeter with excitation at 365 nm and emission at 440 nm.

RESULTS

α-Syn oligomerization was found to be enhanced by the existence of DHA and AA in a dose- and time dependent way, but not that of oleic acid and stearic acid. This result indicate that the existence of .double-bounds in long chain fatty acids is essential for enhancement of α-Syn oligomerization. α-Syn oligomers were found to be positive for SUL and PRL in the sample incubated with DHA, and GLL and HEL in that with AA. BHA and BHT were found to reduce α-Syn ologomerization enhanced by DHA and AA. It was indicated lipid peroxide produced by PUFA formed adduct with α-Syn, then oligomerization and aggregation of the protein.

Free DHA at the concentration higher than 20 μM was found to be cytotoxic to SH-SY5Y cells. Antioxidant tocopherol could not prevent the toxicity of freee DHA. DHA re-incubated with BSA (DHA-BSA) was less toxic than free BSA and tocopherol inhibited the toxicity by DHA-BSA completely.

The amount of PRL, which reflects the level of the proteins conjugated with lipid peroxide derived from DHA, was found to increase in the soluble and insoluble fractions in the cells treated with DHA-BSA. On the othe hand, tocopherol reduced the level of PRL significantly.

These results indicated that the cytotoxic effect of DHA-BSA was induced by the conjugation of oxidation product of DHA with cellular proteins. The cytotoxicity of free DHA should be ascribed to its detergent activity.

DISCUSSION.

In this paper we demonstrated that DHA enhanced oligomerization of α-Syn through adduct formation with lipid peroxide derived from DHA (SUL and PRL) with α-SYN *in vitro*. Using cell

culture system, DHA -BSA, showed weak cytotoxic effect on neural cells and adduct formation of the cellular proteins with PRL was also identified. Antioxidant inhibited cytotoxic effect and adduct formation by DHA-BSA simulataneoously. These results suggest that DHA, which has been believed to be neuroprotective, may become neurotoxic in the condition with increased oxidative stress, such as neurodegenerative disorders.

The increased lipid peroxidation of the membrane is suggested to play a important role in the vicious process in ageing. Lipid peroxides in the membrane initiates sequential reaction of PUFA oxidation to increase the amount of oxidized fatty acids to decrease the membrane fluidity and perturb the function of the proteins associated with cellular membrane. In PD, where dopamine neuron degenerated and membrane-associated protein, α-Syn is aggregated, increased oxidative stress, mitochondrial dysfunction and impaired ubiquitin-proteasome system were observed. Oxidation of PUFA, especially DHA may decrease the binding capacity of α-Syn to the membrane. α-Syn released from the membrane to the cytosol can not stabilize its α-helix structure no more.. In addition, lipid peroxide may directly make adduct with α-Syn and induce protein oligomerization as shown in this paper.

Epidemiological study suggests that the intake of fish oil and vegetable decrease the risk of neurodegenerative disorders, such as Alzheimer disease (AD) and PD (13). However, intervention of neurodegenerative disorders by DHA has not been successful. The results of this paper that in the brain of neurodegenerative disorders, where oxidative stress increased, DHA may neurotoxic. DHA may be effective when it is administrated not after but before the onset of the disease. In addition, intake of the food-derived antioxidant such as polyphenol which can prevent the oxidation of DHA may increase its usefulness (Fig.1). The further investigation to clarify the effect of food-derived DHA and polyphenol using clinically available biomarker is now under the way.

REFERENCES

[1] Carrié, I., Guesnet, P., Bourre, J.M., and Francès, H.: Diets containing long-chain n-3 polyunsaturated fatty acids affect behaviour differently during development than ageing in mice. Br J Nutr., 83, 439-47, 2000

[2] Bazan, N.G.: Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor., J Lipid Res., 44, 2221-2233, 2003

[3] Akbar, M., Calderon, F., Wen, Z., and Kim, H.Y.: Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival., Proc Natl Acad Sci U S A., 102, 10858-10863, 2005

[4] Bazan, N.G.: Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress, Brain Pathol., 15, 159-166, 2005

[5] Katz, R., Hamilton, J.A., Pownall, H.J., Deckelbaum, R.J., Hillard, C.J., Leboeuf, R.C., Watkins, P.A.: Brain uptake and utilization of fatty acids, lipids & lipoproteins: recommendations for future research., J Mol Neurosci., 33, 146-50, 2007

[6] Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L.: Mutation in the alpha-synuclein gene identified in families with Parkinson's Disease. *Science*, **276**, 2045-2047, 1997

[7] Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J.T., Schöls, L., and Riess, O.: Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet.*, **18**, 106-108, 1998

[8] Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D.G., and de Yebenes, J.G.: The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia., *Ann Neurol.*, **55**, 164-173, 2004

[9] Murphy, D.D., Rueter, S.M., Trojanowski, J.Q., and Lee, V.M.: Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons., *J Neurosci*, **20**, 3214-3120, 2000

[10] Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J., and Wolozin, B.: alpha-Synuclein shares physical and functional homology with 14-3-3 proteins. *J. Neurosci.*, **19**, 5782–5791, 1999

[11] Sidhu, A., Wersinger, C., and Vernier, P.: alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease., *FEBS Lett.*, **565**, 1-5, 2004

[12] Kato, Y., Mori, Y., Makino, Y., Morimitsu, Y., Hiroi, S., Ishikawa, T., and Osawa, T.: Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification, *J Biol Chem.*, **274**, 20406-20414, 1999

[13] Schaefer, E.J., Bongard, V., Beiser, A.S., Lamon-Fava, S., Robins, S.J., Au, R., Tucker, K.L., Kyle, D.J., Wilson, P.W., amd Wolf, P.A.: Plasma Phosphatidylcholine Docosahexaenoic Acid Content and Risk of Dementia and Alzheimer Disease. The Framingham Heart Study., *Arch Neurol.* 63:1545-1550, 2006

LEGENDS FOR FIGURE

Fig 1. DHA is generally neuroprotective, but potentially neurotoxic when the oxidative stress is increased.

Under the oxidative stress, DHA may produce toxic lipid peroxide and produce toxic protein adducts. Polyphenol and other food-derived antioxidant might prevent the toxicity of DHA by reducing the oxidative stress.

DA-synthesis enzyme

dopamine

iron

Toxic
lipid peroxide

Neuroprotective
DHA

α-syn

Toxic filamentous syn

Oxidative
modification

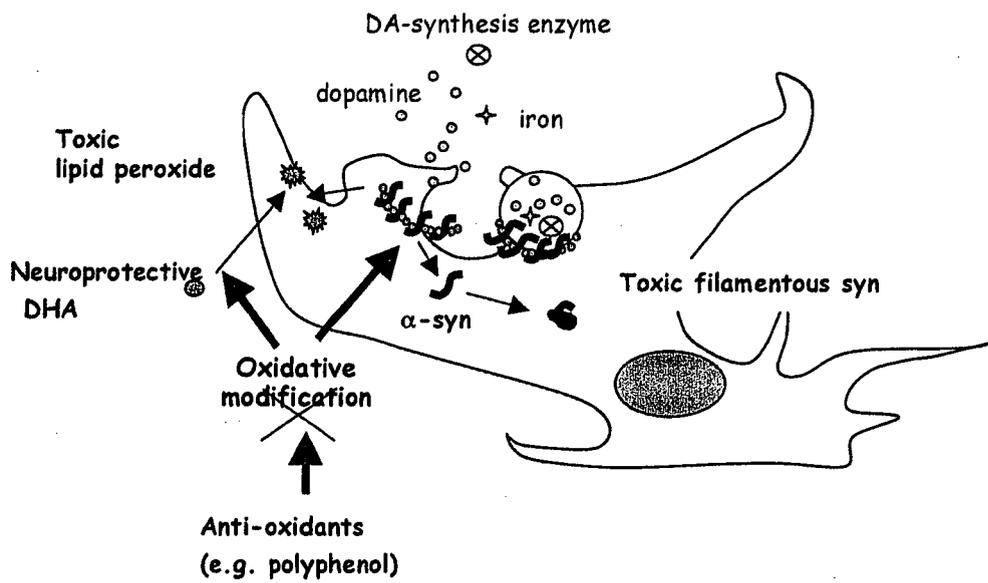Anti-oxidants
(e.g. polyphenol)

Fig.1

Neuromelanin selectively induces apoptosis in dopaminergic SH-SY5Y cells

by deglutathionylation in mitochondria: Involvement of the protein and

melanin component

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Abbreviations used: Cys-DAM, Cysteinyl dopamine melanin; DAM, dopamine-melanin;

DFX, deferoxamine mesylate; EGCG, (-)-epigallocatechin gallate; NM, neuromelanin;

PD, Parkinson's disease; P-K NM, protease K treated neuromelanin; PrS-SG,

S-glutathionylated protein

Abstract

Parkinson's disease is characterized by selective depletion of nigral dopamine neurons

containing neuromelanin, suggesting the involvement of neuromelanin (NM) in the

pathogenesis. This paper reports induction of apoptosis by NM in SH-SY5Y cells,

whereas protease K-treated NM, synthesized dopamine- and cysteinyl-dopamine-melanin

showed much less cytotoxicity. Cell death was mediated by mitochondria-mediated

apoptotic pathway, namely collapse of mitochondrial membrane potential, release of

cytochrome c and activation of caspase 3, but Bcl-2 overexpression did not suppress

apoptosis. NM increased sulfhydryl content in mitochondria, and a major part of it was

identified as GSH, whereas dopamine-melanin significantly reduced sulfhydryl levels.

Western blot analysis for protein-bound GSH demonstrated that only NM reduced

S-glutathionylated proteins in mitochondria and dissociated macromolecular structure of

complex I. Reactive oxygen and nitrogen species were required for the

deglutathionylation by NM, which antioxidants reduced significantly with prevention of

apoptosis. These results suggest that NM may be related to cell death of dopamine

neurons in Parkinson's disease and aging through regulation of mitochondrial redox state

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and *S*-glutathionylation, for which NM-associated protein is absolutely required. The

novel function of NM is discussed in relation to the pathogenesis of Parkinson's disease.

Keywords: neuromelanin, mitochondria, Redox status, Parkinson's disease, apoptosis,

S-glutathionylation.

Running title: Neuromelanin causes apoptosis by modifying SH state

Neuromelanin (NM) is a pigment localized in the catecholaminergic neurons in the substantia nigra, locus coeruleus and other brainstem nuclei. In Parkinson's disease (PD), dopaminergic and noradrenergic neurons are preferentially affected (Hirsch *et al.* 1988), and the presence of NM in most of these neurons suggests that NM may be involved in the cell vulnerability (Kastner *et al.* 1992). NM is known to appear after 2-3 years of life and increase with age to a level of 2.3-3.7 mg/g wet weight of the substantia nigra pars compacta at 50-90 years of age (Zecca *et al.* 2001). A major NM composition is melanin, which is synthesized from quinones and semiquinones, products of autoxidation of dopamine, noradrenalin and L-DOPA, and accounts for about 11-13% of NM (Wakamatsu *et al.* 2003). The melanin composition is a mixture of two melanin classes, eumelanin and pheomelanin in the ratio of 1-3 to 1 (Odh *et al.* 1994). Eumelanin is black, insoluble, and composed of indole monomers derived from oxidized catechol derivatives. Pheomelanin contains about 10% sulfur, and is brownish red, alkaline soluble and produced from 5-S-cysteinyl-dopamine and -DOPA derived by conjugation of dopamine quinone with GSH or cysteine (Odh *et al.* 1994). Recently, studies on the surface oxidation potential of NM suggest a spherical architecture of the

pheomelanin core with eumelanin surface (Bush *et al.* 2006). In addition, protein, lipids

and trace metals are detected in NM, but it has never been clarified whether these

components are integrated in a complex structure of NM, or only associated to melanin in

NM granules. NM contains protein components of about 15% of NM mass (Gerlach *et al.* 1995; Zecca *et al.* 2000). Proteomics of NM granules identified about 70 proteins

occurring commonly in human brain tissues, mainly lysosomal proteins, suggesting

non-selective protein binding to dopamine quinone (Tribl *et al.* 2005). In parkinsonian

brains, α-synuclein, a component of Lewy body was reported to bind to NM or NM

granules, but not in control brain (Fasano *et al.* 2003; Halliday *et al.* 2005). Solid-state

NMR studies indicate the presence of glycidic and aliphatic components attributed to

lipid materials, which corresponds to 20% of NM weight and identified as C_{14}-C_{18} fatty

acids and dolichol, (Zecca *et al.* 2000; Fedorow *et al.* 2005). NM contains also

inorganic components, iron, copper and zinc, as being about 1.5% of NM weight (Zecca

et al. 1994). The high iron content in NM increases vulnerability of NM-containing

dopamine neurons through the production of reactive oxygen and nitrogen species (ROS,

RNS) (Ben-Shachar *et al.* 1991) and of cytokines and nitric oxide in microglia (Wilms *et*

al. 2003).   Recently NM was found to inhibit the ubiquitin-proteasome system through

increased ROS-RNS production, suggesting the involvement of NM in the accumulation

of modified protein in the dopamine neurons (Shamoto-Nagai *et al.* 2004, 2006).

However, NM may be also protective within dopaminergic cells by scavenging free

radical species and binding toxic compounds.   Human NM, but not synthesized melanin,

scavenges hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005).   NM

binds and sequesters redox-active trace metals, dopaminergic neurotoxins, such as MPP^+

(D'Amato *et al.*, 1986) and 1,2(*N*)-dimethyl-6,7-dihydroxy-isoquinolinium ion (Naoi *et al.*

1994), and cytotoxic dopamine quinine (Youdim *et al.* 1994).

Human NM and synthetic melanin from dopamine (dopamine melanin, DAM) or

L-DOPA, induced cell death in rat pheochromocytome PC12 cells (Offen *et al.* 1997) and

primary mesencephalic culture cells (Nguyen *et al.* 2002).   NM and DAM are

phagocytosed into neuronal SK-N-SH, but not to glial U373 cells, and melanin is

localized in the cytoplasm (Li *et al.* 2005).   DAM-induced cell death in PC12 and

SK-N-SH cells was reported to be apoptosis-like, but the detailed mechanism behind the

cytotoxicity remains to be elucidated.   In addition, the involvement of the NM protein

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and melanin species in the cytotoxicity has been scarcely studied.

In PD, the reduction of GSH with corresponding increase of GSSG was confirmed

in the substantia nigra (Riederer *et al.* 1989). GSH plays a major role in the cellular

defense against oxidative stress by direct scavenging ROS-RNS, and GSH depletion

initiates cell death by reduced compensation of oxidative stress. On the contrary,

depletion of GSH protected cells against Fas-mediated cell death (Musallam *et al.* 2002),

and *N*-acetyl-L-cysteine and GSH monoester enhanced hypoxia-induced apoptosis

(Qanungo *et al.* 2004). The cytotoxic effects of GSH may be due to requirement of

reducing conditions to activate caspase 3, an apoptosis executor, by caspase 8 (Hentze *et*

al. 2002), and to suppress nuclear factor (NF)-κB transactivation (Qanungo *et al.* 2004).

Recently, modification of protein thiols by ROS-RNS is gathering attention for its role in

cellular dysfunction *via* reversible formation of mixed disulfides between the protein thiol

and sulhydryl (SH) residue of GSH (PrS-SG) (Maher 2006). This posttranslational

modification of protein, referred as *S*-glutathionylation, regulates the essential cellular

functions, such as energy synthesis, signal transduction pathway and transcriptional

activation. (Schafer and Buettner 2001). Considering that NM contains SH groups, it

should be clarified whether NM can affect S-glutathionylation in mitochondria and

regulate the redox status, which might be involved in the selective death cascade of

NM-containing dopamine neurons.

In this paper, the cytotoxicity of NM was examined in human dopaminergic

neuroblastoma SH-SY5Y cells using NM prepared from human substantia nigra. To

examine the roles of the protein and melanin composition in the cytotoxicity, NM was

treated with proteinase K (P-K NM), and eumelanin and pheomelanin were synthesized

by oxidation of dopamine in the absence (dopamine melanin, DAM) or presence of

L-cysteine (cysteinyl-dopamine melanin, Cys-DAM). Wild and transfection-enforced

Bcl-2 overexpressed SH-SY5Y cells (Bcl-2 cells) (Akao et al. 2002) and mitochondria

prepared from them were used to clarify the apoptotic process. The effects of NM on

intracellular redox and SH states were studied to clarify the relation of

S-glutathionylation to cell death process. The involvement of NM in the pathogenesis

of PD is discussed, where increased oxidative stress, mitochondrial dysfunction and

induction of apoptosis are proposed to induce selective degeneration of dopamine

neurons containing NM.

Materials and methods

Materials

3,3'-Dihexyloxcarbocyanide iodide [DiOC$_6$(3)], LIVE/DEAD viability/cytotoxicity assay

kit for mammalian cells and Measure-iT^{TM} thiol assay kit were purchased from Molecular

Probes (Eugene, OR, USA); superoxide dismutase (SOD), deferoxamine mesylate (DFX),

(-)-epigallocatechin gallate (EGCG) were from Sigma (St. Louis, MO, USA). A

substrate for caspase 3, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid

α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA), was obtained from Peptide Institute

(Osaka, Japan). Catalase from bovine liver, Dulbecco minimum essential medium

(DMEM) and other reagents were from Wako (Kyoto, Japan). SH-SY5Y cells were

cultured in Cosmedium-001 tissue culture medium (CosmoBio, Tokyo, Japan),

supplemented by 5% fetal calf serum in 95% air and 5% CO$_2$. Bcl-2 was overexpressed

in SH-SY5Y cells (Bcl-2 cells) by transfection as reported previously (Akao *et al.* 2002).

Preparation of NM and DAM