quantitatively analyzed by FACS and apoptotic cells were detected as the subG$_1$ peak (Fig. 1 B). NM treatment increased the number of apoptotic cells significantly to 37.9% of the total cells from 2.23% in control, whereas DAM induced apoptosis in 11.6% cells. Fig. 1 C shows the effects of NM and DAM concentrations on the viability of the wild and Bcl-2 cells as measured quantitatively using FACS. NM at concentrations higher than 10 μg/ml induced apoptosis in the wild cells in a dose-dependent way. Transfection-enforced Bcl-2 overexpression did not prevent apoptosis caused by NM and DAM.

Effects of melanin species and protein component of NM on the cytotoxicity

NM contains protein component in addition to mixed melanin of black eumelanin and brown pheomelanin. SH residues were detected in NM as measured with the fluorometric Thiol assay kit, which can detect GSH, cysteine and related SH compounds in free and protein-bound form. SH content in NM was determined to be 2.42 ± 0.80 nmol/mg melanin, whereas SH was not detected in DAM (Table 1). Protease K treatment of NM reduced the SH contents significantly to 0.57 ± 0.16 nmol/mg melanin,

23% of the un-treated NM. In Cys-DAM synthesized from dopamine in the presence of L-cysteine, high SH content was determined, 3.77 ± 0.19 nmol/mg melanin.

The cytotoxicity of these melanin species was quantitatively measured by calcein staining for live cells (Fig. 2 A). Among 4 melanin classes, only NM reduced the number of live cells, and the protease-K treatment suppressed the NM cytotoxicity. DAM and Cys-DAM were much less cytotoxic than NM and the difference from control was not statistically significant. The cell death was confirmed by histopathological observation after staining with Hoechst 33342 (Fig. 2 B). NM and dopamine induced apoptosis with the typical condensation and fragmentation of nucleus in most of the cells, while P-K NM, DAM and Cys-DAM virtually did not induce cell death.

Apoptosis pathway activated by NM

Mitochondria were prepared from the wild and Bcl-2 cells, treated with NM and then subjected to FACS analyses after stained with $DiOC_6(3)$. Fig. 3 A shows that NM reduced $\Delta\Psi m$ of the wild cells in a dose-dependent way, which GSH did not prevent. In mitochondria prepared from Bcl-2 cells, the reduction of $\Delta\Psi m$ by NM was also observed

(Fig. 3 B).

After treated with NM, cytochrome c was released from mitochondria into cytosol in wild SH-SY5Y cells in a time-dependent way (Fig. 3 C). The cells were treated with NM, DAM and dopamine for 16 h, and the activity of caspase 3 was measured fluorometrically. Fig. 3 D shows the significant increase in caspase-3 activity in the wild cells treated with NM and dopamine. On the other hand, DAM treatment did not affect the activity. In Bcl-2 cells, increase in caspase 3 activities was much less than in the wild cells, but NM still increased the activity markedly. Bcl-2 overexpression completely prevented the increase in caspase 3 activity by dopamine.

Effects of NM and DAM on SH state in mitochondria

Effects of NM and DAM on mitochondrial SH levels were examined. Mitochondria prepared from the wild and Bcl-2 cells were incubated with NM (25 μg/ml) and SH contents were measured for 3 h, and NM significantly increased SH levels in mitochondria from 30 min to 2 h and reached to a plateau (Fig. 4 A). On the other hand, DAM (25 μg/ml) and dopamine (100 μM) significantly reduced SH contents in a

time-dependent way (Fig. 4 B). The effects of four melanin species on mitochondrial SH levels are shown in Fig. 4 C. P-K NM did not increase SH, while DAM reduced SH after 2 h incubation. The increased SH residues were identified to be GSH by use of HPLC, as shown in Fig. 4 D. NM significantly increased GSH levels in mitochondria, whereas P-K NM did not affect and DAM reduced GSH levels, as in the case of the total SH contents measured by the fluorescent assay.

To confirm the localization of NM-increased SH compounds in sub-mitochondrial fractions, mitochondria were treated with these melanins, and differentiated into the soluble fraction and the precipitate. After NM-treatment, the SH contents increased significantly in both the fractions, whereas DAM and especially dopamine reduced SH contents (Fig. 5 A). Cys-DAM markedly increased SH in the soluble fraction and pellets.

NM- and DAM-treated cells were fractionated into mitochondria and cytosol fraction, and GSH contents were quantified by the enzyme-recycling method (Fig. 5 B). NM significantly increased GSH in mitochondrial and cytosol fraction, but DAM did not affect GSH levels.

Table 2 summarizes the total SH, GSH and GSSG contents, and the GSSG/GSH ratio in the wild cells, the subcellular fractions and the isolated mitochondria after NM- and DAM-treatment. NM increased the total SH and GSH contents in the cells, the mitochondria fraction and the isolated mitochondria, significantly ($p < 0.05$), but not in the cytosol. GSSG levels were also increased in these samples after treated with NM. DAM reduced the total SH and GSH contents and increased GSSG levels in the mitochondrial fraction and the isolated mitochondria. NM increased significantly the GSSG/GSH ratio in the cell lysate, mitochondrial fraction and isolated mitochondria and DAM increased the ratio more markedly.

Effects of antioxidant on the cytotoxicity and SH reduction by NM

The involvement of ROS-RNS in NM-induced cell death and increase of mitochondrial SH levels was examined. After incubated with NM in the presence of antioxidants, the cell viability was quantitatively measured by calcein staining. Iron-chelating DFX, SOD and nitric oxide (NO)-scavenging EGCG protect cells from cell death induced by NM, but catalase did not (Fig. 6 A). At the same time, DFX, SOD and EGCG prevented

the NM-induced increase in mitochondrial SH contents, but catalase further increased SH levels (Fig. 6 B).

The effects of NADPH-dependent recycling system on the NM-increased SH levels were examined in mitochondria. NADPH enhanced NM-induced increase in SH levels, but did not affect the levels in control and DAM-treated mitochondria.

Effects of NM on *S*-glutathionylated protein (PrS-SG) in mitochondria

To find the origin of GSH increased in mitochondria by NM, mitochondria prepared from the wild cells were treated with NM and other melanin (10 μg/ml), and the protein was subjected to Western blot analysis for *S*-glutathionylated protein (PrS-SG). Under non-reducing conditions PrS-SG was detected in mitochondria without NM treatment (Fig. 7 A). NM reduced ProS-SG especially with high molecular mass, while other melanin increased PrS-SG. Under reducing conditions, reduction of PrS-SG was confirmed again in NM-treated mitochondria, where PrS-SG proteins with molecular mass higher than 50 kDa were reduced significantly (Fig. 7 B). Mitochondrial complex I proteins were visualized with anti-complex I antibody. Under non-reducing conditions,

NM treatment disaggregated the macromolecular structure of complex I. Complex I proteins with 100-150 kDa disappeared, and complex I subunits with less than 75 kDa increased significantly. On the other hand, other melanin did not affect the high structure of complex I. Under reducing condition the amount and electrophoresis-pattern of complex I subunits did not change by NM-treatment, as shown by Western blot analyses, indicating the reversibility of NM-induced dissociation of complex I subunits. Under non-reducing conditions, the reactivity against anti-complex III antibody was slightly reduced by NM treatment, but other melanin did not affect the amounts and pattern of complex III subunits. In mitochondria prepared from Bcl-2 cells, the same results were obtained.

Discussion

This paper presents a novel role of NM in the pathogenesis of PD. NM induces apoptosis in SH-SY5Y cells through activation of death cascade, which depends on the protein component, SH content and melanin species. In PD NM contents in the substantia nigra reduced to 1.2-1.5 mg/g wet weight from 2.3-3.5 mg/g wet weight (Zecca

et al. 2004), as indicated by loss of dark brown color in this region. However, it remains unclear whether the protein, lipid and inorganic components of NM change the nature in PD. Protein associated with NM granules from normal brains was subjected to the proteomic studies and about 70 kinds of protein were detected, but the accumulation of specified proteins was not confirmed (Tribl *et al.* 2005). In PD brain, α-synuclein is associated with NM (Fasano *et al.* 2003), and NM isolated from PD brains is composed mainly of highly cross-linked, protease resistant protein-like materials (Aime *et al.* 2000). Considering the increase of oxidative stress in parkinsonian brains, proteins in NM granules might be highly modified with ROS-RNS and tend to be more aggregated, which may be accelerated further by dysfunction of the ubiquitin-proteasome system (McNaught *et al.* 2001). However, the cytotoxicity of NM-associated protein itself has never been reported in NM prepared from either normal or parkinsonian brains. In A9 neurons of PD brains, the loss of cholesterol and the aggregation of α-synuclein to lipid in NM were reported by histopathological observation (Halliday *et al.* 2005). However, the lipid components may not be involved in the toxicity of NM observed in this paper, since lipid is washed out during the purification procedure (Double *et al.* 2000).

The role of protein component in the NM toxicity was clearly demonstrated in this paper. Protease-K treatment totally reduced NM cytotoxicity, and synthesized DAM and Cys-DAM did not induce the cell death in SH-SY5Y cells under used conditions. In NM, SH residues were detected as exposed on the surface, which protease K-treatment reduced to one fourth, suggesting that a major part of SH groups is derived from the protein and the rest from the pheomelanin component. This result may be comparable with the previous result that protease K-treatment reduced amino aid contents from 165 to 57 μg/mg melanin (Double *et al.* 2000). According to the reported amino acid composition, the cysteine content of NM is 10.6 ± 3.7 nmol/mg melanin (Double *et al.* 2000). SH level in NM is quantified to be 2.42 ± 0.80 nmol/mg melanin, suggesting that most of cysteine is sequenced in protein as intra- or inter-disulfide bond, or occurs as the mixed disulfide bond between protein SH and GSH, cysteine or related SH derivatives. In pheomelanin produced from 5-S-cysteinyl-dopamine a conjugate of cysteine with *o*-quinone, free SH residues were detected, even though pheomelanin has been considered to polymerize into a benzothiazine structure.

NM and DAM affected SH state in mitochondria in quite opposite ways. NM and

Cys-DAM increased SH contents in mitochondria, whereas DAM and dopamine reduced them markedly. These results might be comparable to the previous results that DAM increased ROS-RNS and induced cell death in neuronal SK-N-SH cells, whereas NM protected the cells from hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005). Melanosomes containing eumelanin and pheomelanin have oxidation potentials of - 0.2 and + 0.5 V, respectively (Samokhvalov *et al.* 2005). Pheomelanin in NM and Cys-DAM may reduce disulfide bonding and release GSH or cysteine from the mixed disulfide bonding and increases SH content in mitochondria, while eumelanin and dopamine oxidatively modify SH residues. These results are relevant with the fact that NM has the surface oxidation potential different from synthesized DAM (Bush *et al.* 2006).

The role of NM-associated protein was examined in concern to regulation of mitochondrial "redox state", which is related to many physiological and pathological phenomena of cells (Schafer and Buettner, 2001; Mahler 2006). Redox state depends on the reduction potential of redox pairs, GSH/GSSG, reduced/oxidized thioredoxin [$Trx(SH)_2$/TrxSS] and NADPH/$NADP^+$. GSH/GSSG pool is the largest in cells and a

major indicator of the redox state. Most glutathione is localized in the cytosol, but there is a GSH pool in mitochondria, which accounts for about 15% of the total (Meister 1995). GSH reduces thiol modifications of disulfide bonding and thioesters, and it is a substrate of protein *S*-glutathionylation. *S*-Glutathionylation is reversible and transitional in cytosol, where mM order of GSH and ascorbic acid are present, but in mitochondria with high oxidative environments (Table 2), *S*-thiolated proteins are more stabilized and detected in complex I, as reported here and by Taylor *et al.* (2003). Glutathionylated $NADP^+$-dependent isocitrate dehydrogenase was detected in brains from a PD model of MPTP-treated mice (Kil and Park, 2005). Under oxidative conditions, actin, glyceraldehyde-3-phosphate dehydrogenase, protein kinase, HSP27, protein-tyrosine phosphatase 1B, protein kinase Cα are the substrate of *S*-thiolation (Eaton *et al.* 2002). In physiological conditions or mild oxidative stress, thiols in protein (PrS-SG) or GSH are reversibly modified into active intermediates, such as thiolate, sulfenate and sulfenic acid, by NO, superoxide, hydrogen peroxide and peroxynitrite (Klatt and Lamas, 2000). The activated protein SH groups reacts with GSH, or *vice verse* activated GSH reacts with reduced protein SH to generate GSH-protein mixed disulfide (PrS-SG). Prolonged

or intense generation of ROS-RNS irreversibly oxidized thiols into protein cysteic acid by sulfonation, which was detected in the brains of patients with PD and Alzheimer' diseases (Choi *et al.* 2005). Glutathionylation is reversed by glutaredoxin (GRX), other thioredoxin (TRX) and protein disulfide isomerase [EC 5.3.4.1], and yields free SH in protein (Pr-SH) and GSH from PrS-SG. This reaction is recycled by TRX reductase [EC 1.6.4.5] or GSH reductase [EC 1.6.4.2] using NADPH as a cofactor.

NM significantly reduces *S*-glutathionylated proteins detected in mitochondria, especially in complex I (Fig. 7). In addition, NM dissociates high structure of complex I into the subunits, and the dissociation is reversed by reducing agents, suggesting that *S*-glutathionylation stabilizes the high structure of complex I under physiological condition. It may be reasonable to consider that SH group of NM (NM-SH) functions as that in GRX, reduces the disulfide bonding in mitochondrial protein, release free GSH or cysteine and exposes free protein SH. Only NM, but not Cys-DAM, reduces mitochondrial PrS-SG, indicating that NM-associated protein, not pheomelanin, affects the mixed disulfide bonding in mitochondria. The SH amounts in NM used for these experiments were less than 1% of the total SH content present in mitochondria,

suggesting the "catalytic" function of NM. NADPH increased SH release by NM, but did not affect SH content in control, suggesting that NM recycling by NADPH enhances the deglutathionylation in a similar way as GRX.

The mechanism underlying induction of apoptosis by NM requires further studies to be fully elucidated. NM activated "intrinsic" apoptotic pathway, but Bcl-2 overexpression did not prevent cell death, even though Bcl-2 protects the cells against apoptosis induced by dopamine (Fig. 3 D) and a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol (Maruyama *et al.* 2000, Akao *et al.* 2002). NM may activate inner membrane anion channel through the altered redox state depending on the GSH/GSSG ratio (Aon *et al.*, 2007), or the modification of vital SH in mitochondria. Diazenedicarboxylic acid bis 5*N*,*N*-dimethylamide (diamide) induced mitochondrial permeability transition (mPT) by modifying thiol of Cysteine-57 in adenine nucleotide translocator (ANT) localized on the matrix site at the cyclosporine-binding site (Costantini *et al.* 2000). Modification of Cysteine-57 in ANT with NO could prevent mPT, maybe by protection of the vital SH against further cytotoxic modification. EGCG, a NO scavenger, and SOD prevented the NM-induced apoptosis and increase in

mitochondrial SH, suggesting the involvement of NO, superoxide and peroxynitrite. In addition, iron released from NM may enhance ROS-RNS production, as shown by the protection of DFX. NM may remove protective GSH or cysteine from mixed disulfide bonds in mPT pores and expose vital SH to subsequent modification by ROS-RNS, resulting in induction of mPT and apoptosis, which overcomes the protection by Bcl-2.

As a conclusion, this paper clearly presents data that NM induces apoptosis in SH-SY5Y cells by means of the protein component. The mechanism is clarified as that NM deglutathionylates specified PrS-SG in mitochondrial complex I, dissociates the higher structure of mitochondria, causes the dysfunctions, and finally activates apoptosis signaling. These effects of NM on the redox state were the most manifest in isolated mitochondria, where GSH is not produced *in situ* and the level is mainly regulated by GSH recycling system composed of NADPH-dependent reductase. These situations in isolated mitochondrial may reproduce those in the substantia nigra of aged and parkinsonian brains, where the redox state tends to more oxidizing condition with reduced GSH levels. In normal condition the compensative antioxidant capacity might prevent the activation of death process by NM, even though it accumulates in the

Comment [mn1]:

substantia nigra at the concentrations of 2-4 μg/mg wet weight, almost the same levels as used in this paper, 10-25 μg/ml of reaction mixture. Further studies will clarify the more detailed role of NM in the malignant cycles between oxidative stress, mitochondrial dysfunction cell death of dopamine neurons in PD and aging. In addition, a quite new strategy may be found to prevent or delay the cell death itself by stabilization of mitochondrial redox state and *S*-glutathionylation.

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