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Apoptosis in dopaminergic neurons of the human substantia nigra during normal aging

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Summary. Morphological and biochemical alterations have been described in neurons of the aged human brain. However, the cell death process associated with neuronal senescence remains to be elucidated. Apoptosis and autophagic degeneration, two modes of programmed cell death described in embryogenesis and tissue renewal in adult, have been observed in nigral dopaminergic neurons in patients with Parkinson's disease. In the present study, we made the hypothesis that programmed cell death may be also involved in the death of nigral dopaminergic neurons occurring during aging. Cell death types were defined by morphological criteria identified at subcellular level. We thus performed an ultrastructural analysis in order to search for apoptotic and autophagic features in melanized neurons of the substantia nigra in four normal aged subjects. Morphological characteristics of apoptosis, such as contact loss with surrounding tissues, cell shrinkage and chromatin condensation, were found in 2% of the total number of melanized neurons analyzed. Although endoplasmic reticulum appeared normal, mitochondria were markedly shrunken. Fragments of melanized neurons were found in glial cells. Autophagic degeneration or necrosis were not detected in melanized neurons. Signs of oxidative stress, such as vacuolation of mitochondria, were observed in melanized neurons devoid of apoptotic features. These findings demonstrate that apoptosis is involved in cell death of nigral dopaminergic neurons during normal aging. Since morphological abnormalities found in this study, such as marked mitochondrial shrinkage in apoptotic neurons, were not observed in patients with Parkinson's disease, the mechanisms underlying apoptosis may be different in aging and pathology.

Key words: Aging, Apoptosis, Dopaminergic neurons, Substantia nigra, Ultrastructural analysis

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Introduction

Senescence is a natural process that results, at cellular level, in decreased metabolism activity (Kanungo, 1994). Although several hypotheses, such as mitotic clock linked with chromosome ends, expression of specific genes or increasing oxidative stress (Kanungo, 1994), have been proposed, the cause and basic mechanisms of aging remain to be elucidated. Alterations in the expression of numerous genes, leading to the diminution in the levels of various enzymes and hormones, have been observed during aging both in pre- and post-mitotic cells. For example, the expression and activity of superoxide dismutase and catalase, two oxidant-free radical scavengers, are lowered in the brain of the old rat (Semsei et al., 1991), whereas the expression of calbindin-28 kD, involved in calcium buffering and intraneuronal calcium homeostasis, is decreased in aging human brain (Iacopino and Christakos, 1990). Neuronal loss has been reported in several regions of the senescent brain in human, such as cerebrum and cerebellum cortices, hippocampus, nucleus basalis of Meynert, locus coeruleus and substantia nigra (Anderson et al., 1983; Kemper, 1984). Decreases in nuclear area and nucleolar volume, increase in nuclear envelope length, moderate disorganization of the endoplasmic reticulum and mitochondrial vacuolation have been detected in the perikarya of senescent neurons (Adams and Jones, 1987). Dendritic arborization of neurons undergoes changes related with aging in various regions of the brain. For example, decreased length of dendritic processes and decreased number of branches and dendritic spines have been described in substantia nigra (Cruz-Sánchez et al., 1995). Despite the characterization of biochemical and morphological changes occurring in neurons during aging, the final step of neuronal degeneration, i.e. the cell death process, remains unknown.

Apoptosis, a common type of programmed cell death, is a physiological cell death involved in the developmental program and renewal of tissues in adult (Kerr et al., 1972; Bellamy et al., 1995). It may be

regarded as a controlled process of cell deletion involving an active role played by the degenerating cells. Some of the genes specifically involved in apoptosis in mammals have been identified. Among these genes, *bcl-2* inhibits the process (Ellis et al., 1991), whereas the gene that codes for interleukin-1ß-converting enzyme (ICE) is required for cell death (Yuan et al., 1993). DNA fragmentation is observed frequently, but not always, in cells undergoing apoptosis (Cohen et al., 1992; Oberhammer et al., 1993; Bellamy et al., 1995). Apoptotic cells often lose their contact with surrounding cells and undergo a well defined series of changes, chromatin condensation, shrinkage of cytoplasm in which organelles remain intact and final absorption by phagocytes with no inflammatory reaction (Bellamy et al., 1995). This is in contrast with the passive role of cells undergoing necrosis, another type of cell death generally occurring after acute stress or injury. Necrosis, accompanied by inflammatory reaction, is characterized by cytoplasmic vacuolation, whereas the nucleus remains unchanged, cell swelling and bursting. Other modes of programmed cell death have been described during embryogenesis or experimental models of cell death, such as autophagic degeneration, a process characterized by autodigestion of the cytoplasm by lysosomes (Clarke, 1990; Charriaut-Marlangue et al., 1996).

The relation between programmed cell death and normal aging has been suggested by a few data, such as detection of apoptosis in the tissue regression occurring during menopause (Bardon et al., 1987). Moreover, increased frequency of cells with DNA fragmentation has been observed in the striatum of old rats (Zhang et al., 1995). In neurodegenerative disorders, the cell loss that affects specific neurons in the brain is progressive but more rapid than in aging. The main anatomical characteristic of Parkinson's disease is a massive loss of dopaminergic (DA) neurons in the substantia nigra. Apoptotic and autophagic DNA neurons have been observed in the substantia nigra of parkinsonian patients, indicating that, in Parkinson's disease, cell demise may occur through at least two different modes of cell death (Anglade et al., 1997; Tompkins et al., 1997). Moreover, DNA fragmentation has been detected in degenerating nigral DA neurons of parkinsonian patients (Tompkins and Hill, 1995). The mechanisms involved in this process may be analogous to those described in programmed cell death since *bcl-2* expression has been detected in nigral DA neurons both in normal subjects and parkinsonian patients (Vyas et al., unpublished results). The occurrence of an apoptotic process in other degenerative disorders, such as Alzheimer's and Huntington's diseases, was also suggested by in situ detection of DNA strand breaks (Su et al., 1994; Lassmann et al., 1995; Portera-Caillau et al., 1995). Whether apoptosis is also involved in neuronal death related with senescence remains to be elucidated. In this study, we propose the hypothesis that the DA neurons of the substantia nigra are dying through apoptosis during aging. Since identification of cell death types rests on morphological criteria defined at subcellular level (Schweichel and Merker, 1973; Clarke, 1990; Bellamy et al., 1995), we searched characteristic ultrastructural features of apoptosis in the DA neurons of the substantia nigra in normal aged subjects.

Materials and methods

Subjects

The substantia nigra of four subjects deceased without known neurodegenerative disorder was used in this study. The mean age and postmortem delay before tissue fixation were 82.7±4.7 years and 8.5±1.5 hours (mean±SEM), respectively. Delay between death and tissue fixation did not exceed 12 hours to minimize artifacts due to postmortem degradation. The absence of neurofibrillary tangle or senile plaques after examination of cerebral cortex by Bodian silver impregnation confirmed that the subjects had no pathology related to senile dementia. Clinical and pathological characteristics of the subjects are given in Table 1.

Ultrastructural analysis

After autopsy, the brains were removed from the

Table 1. Number of apoptotic melanized neurons among the total number of melanized neurons with visible nuclei analyzed in the substantia nigra of four normal aged subjects. In subjects 2 and 3, the analysis was performed in the cerebral hemisphere contralateral to the cerebral infarction.

SUBJECTS	AGE (yrs)	PATHOLOGY	CAUSE OF DEATH	POSTMORTEM DELAY (hrs)	TOTAL NUMBER OF MELANIZED NEURONS WITH VISIBLE NUCLEI ANALYZED	NUMBER OF APOPTOTIC MELANIZED NEURONS
1	88	Cardiovasculatory deficiency	Pancreatitis	6	154	2
2	85	Cerebral infarction	Pneumopathy	10	176	5
3	89	Pneumopathy, cerebral infarction	Cardiac failure	6	46	-
4	69	Cardiovasculatory deficiency, cancer of kidney	Cardiac failure	12	34	2

skull and hemisected. Brainstems were cut in transverse slabs of 0.5 cm in thickness and small blocks of the rostral tier of the substantia nigra pars compacta (0.5 x 0.5 x 0.5 cm) were dissected out. The blocks were fixed for 3 days at 4 ºC in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M Na-phosphate buffer and stored in a solution of 0.2M Na-phosphate buffer-0.1% Na-azide. Sections of 200 μm in thickness were cut on a Vibratome. Small pieces of tissue which included melanized DA neurons were selected and removed under a dissecting microscope. Tissues were post-fixed in 2% osmium tetroxide in 0.01M phosphate buffered saline for 30 min and rinsed in distilled water. Dehydration was performed in a graded series of alcohol solutions containing saturated phosphotungstic acid. Embedding of tissue was made in Araldite resin. Semi-thin sections (0.5 μm-1 μm) were cut and stained by toluidine blue to observe accurately the nuclei and cytoplasms of melanized neurons. Ultrathin sections (100-200 nm) were made at this level including neurons with abnormal features. The sections were observed under a JEOL 1200 EX electron microscope at 70 kV.

Results

Melanized neurons with morphological features of apoptosis were detected in three subjects. They represented 2% of the total number of melanized neurons analyzed (Table 1). These neurons had chromatin condensation, increased convolution of nuclear envelop and marked shrinkage of the cytoplasm in which endoplasmic reticulum retained normal morphology (Fig. 1). Condensed chromatin appeared as diffuse aggregates and small patches homogeneous in size (a few hundred nanometers) and evenly distributed in the nucleus of the majority of the neurons. No mass of chromatin larger than 2 μm was detected in the dying cells. In all the apoptotic neurons, most mitochondria were shrunken with increased electron density (Fig. 1A-E), though abnormal mitochondria were less numerous in subject 1 (Fig. 1A-C). Mitochondria retained a normal appearance in the surrounding tissue (Fig. 1B). Few lipid droplets were found in the cytoplasm of apoptotic neurons. The dying neurons had partially or totally lost their contact with surrounding cells (Fig. 1A, D, F). No evidence for engulfment of intact neurons in glial cells could be found in this analysis. Cell fragmentation was detected in one melanized neuron that was not engulfed in glial cell (Fig. 1F). Only fragments of melanized neurons have been observed in the cytoplasm of glial cells (Fig. 1G).

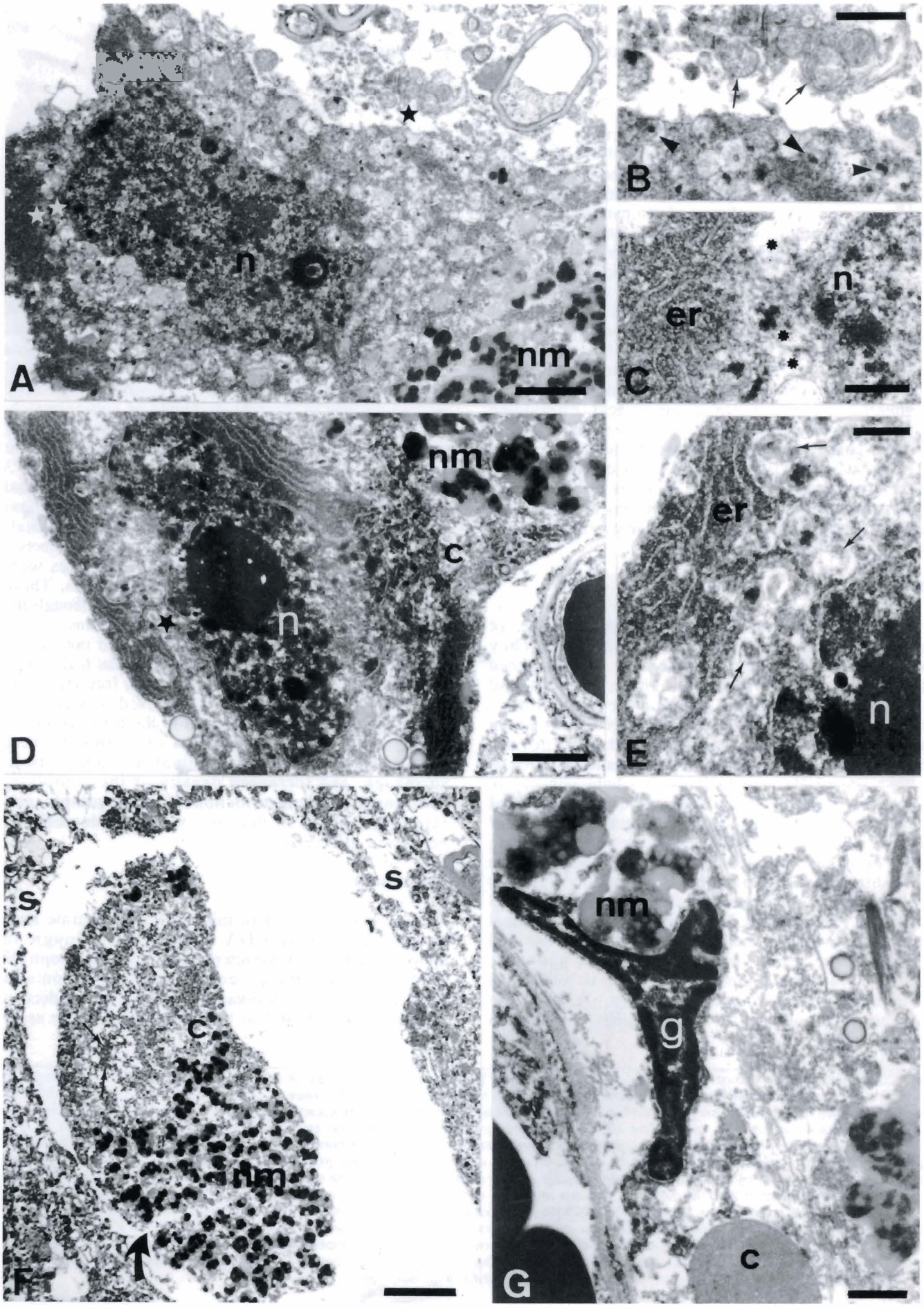
Morphological abnormalities were detected in melanized neurons devoid of apoptotic features. Vacuolation of mitochondria was observed in neurons with intact nucleus and endoplasmic reticulum in all the subjects, though it was more obvious in the oldest subjects (subjects 1 and 3) (Fig. 2A-D). Mitochondria were swollen, with sparse crests and flocculent material in their matrix (Fig. 2D). By contrast, mitochondria of the surrounding cells appeared normal (Fig. 2D). Accumulations of straight filaments of approximately 10nm in diameter were observed in the cell bodies of melanized neurons (Fig. 2E). These aggregates of tight filaments were detected more frequently in subject 3 than in the other subjects. Occasionally, thin (5 nm) and thick (15-16 nm) filaments were found in the cytoplasm of melanized neurons, loosely associated in small bundles. These bundles were observed only in subjects 1 and 3. Inclusions resembling Marinesco bodies were detected in the nucleus of melanized neurons. These neurons had no other striking abnormalities, though the nucleus was slightly convoluted in some neurons.

The majority of melanized neurons did not exhibit morphological abnormalities. These neurons had a large nucleus with small patches of chromatin forming a fine network and mitochondria interspersed between thin tubules of endoplasmic reticulum in the cytoplasm (Fig. 2A, C). Occasionally, features of apoptosis were observed in glial cells: chromatin condensation, shrinkage, and increased density of the cytoplasm containing stacked mitochondria and endoplasmic reticulum. Signs of necrosis were not detected in neurons or glial cells.

Discussion

The data obtained in this work demonstrate that, during aging, nigral DA neurons die through an apoptotic process. Characteristic features of apoptosis detected in the dying neurons, such as chromatin condensation, cell shrinkage and apoptotic bodies in glial cells, are similar to those described in the same

Fig. 1. Apoptosis in dopaminergic neurons containing neuromelanin (nm) in the substantia nigra of normal aged subjects at different stages of degeneration. **A.** Apoptotic neuron with chromatin condensation in the nucleus (n) and shrinkage of cell volume. Partial loss of contact with neighbouring cells is visible in the left part of the field. Bar: 2μm. **B.** Enlargement of the area indicated by a star in A. Most mitochondria located in the neuron are shrunken (arrowheads), whereas the mitochondria of the surrounding tissue have thin crests and normal size (arrows). Bar: 1μm. **C.** Area indicated by two stars in A viewed at higher magnification. The endoplasmic reticulum (er) retains a normal appearance, whereas the mitochondria (asterisks) are more or less shrunken. Condensation of the chromatin is visible in the nucleus (n). Bar: 250nm. **D.** Apoptotic neuron with chromatin condensation in the nucleus (n) and shrinkage of the cytoplasm (c). The empty spaces around the neuron suggest contact loss with surrounding tissue. Bar: 2μm. **E.** Enlargement of the area indicated by a star in D. The endoplasmic reticulum (er) has no striking abnormal features, whereas the mitochondria (arrows) are shrunken. Chromatin condensation is observed in the nucleus (n). Bar: 250nm. **F.** Apoptotic neuron in late stage of degeneration. Despite an advanced degradation process, apoptosis is attested by remains of condensed nuclear chromatin (small arrows) and marked shrinkage of the cytoplasm (c). Maximal contact loss with surrounding cells (s) results in the appearance of a large empty space around the neuron. Beginning of cytoplasmic fragmentation is indicated by a curved arrow. Bar: 4μm. **G.** Clusters of neuromelanin granules (nm) and cell debris resembling clumps of condensed chromatin (c) are observed in the cytoplasm of a glial cell (g). Bar: 1μm.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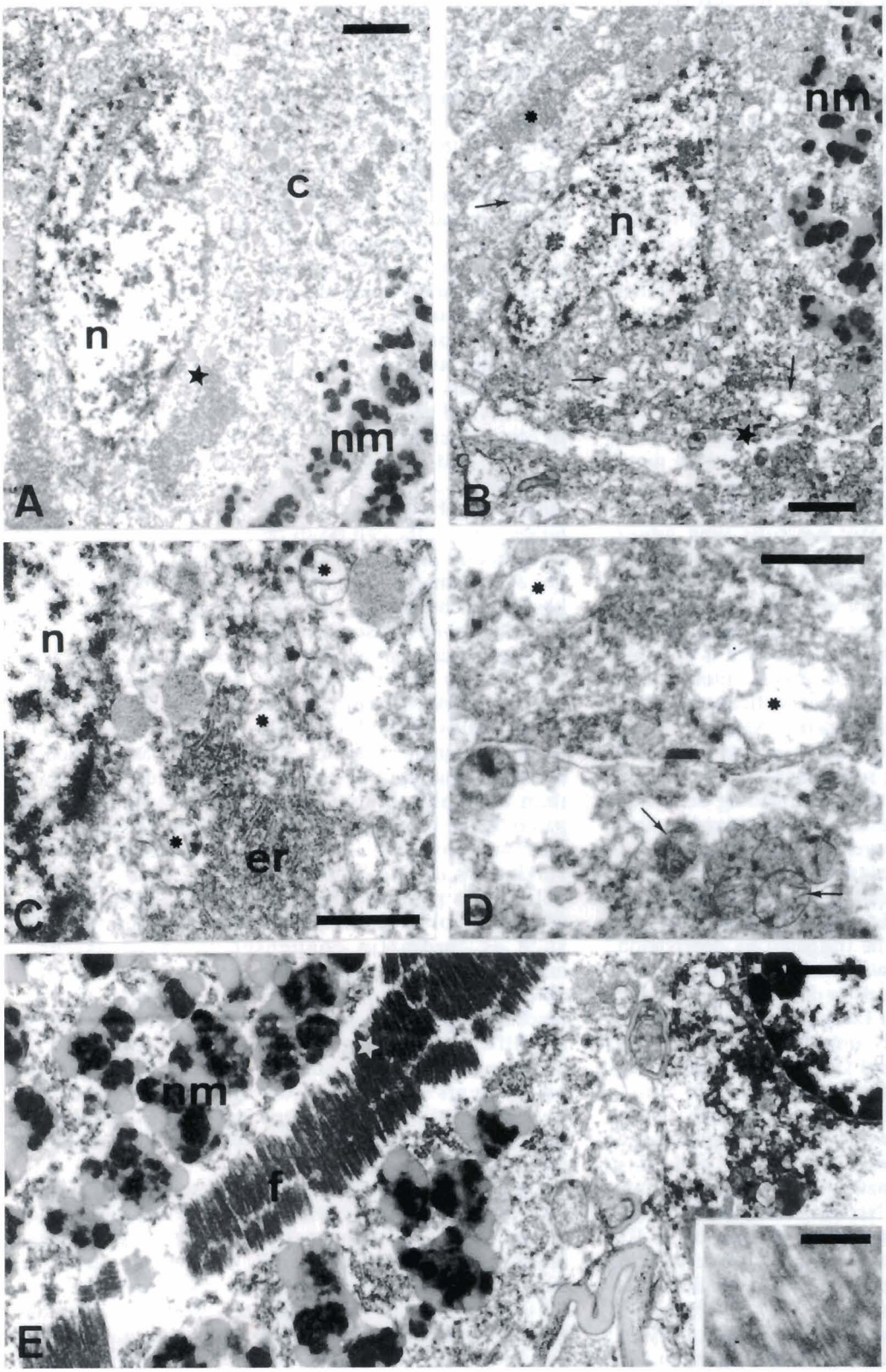

Fig. 2. Abnormal morphological features in non apoptotic dopaminergic neurons containing neuromelanin (nm) in the substantia nigra of normal aged subjects. **A.** Nigral dopaminergic neuron with normal morphology. The nucleus (n) has a characteristic pattern of chromatin, i.e. small patches and filamentous aggregates forming a fine network. No abnormal feature can be detected in the cytoplasm (c) at this magnification. Bar: 2μm. **B.** Nigral dopaminergic neuron with normal nucleus (n). In the cytoplasm, most mitochondria are swollen (arrows), whereas no alteration of endoplasmic reticulum (asterisk) is detectable at the present magnification. Bar: 2μm. **C.** Enlargement of the area indicated by a star in A. The endoplasmic reticulum (er) and mitochondria (asterisks) have normal morphology. Small patches of chromatin are observed in the nucleus (n). Bar: 1μm. **D.** Area indicated by a star in B viewed at higher magnification. The mitochondria (asterisks) in the cytoplasm of the neuron are swollen and their crests hardly visible. In contrast, the mitochondria in the adjacent tissue have normal morphology with thin crests (arrows). Bar: 1μm. **E.** Filamentous accumulation (f) that forms a «lattice network» in the cytoplasm of a neuron containing neuromelanin (nm). The insert is an enlargement of the small zone indicated in E by a star. The periodic structure visible at this magnification is suggestive of a tight association of filaments of approximately 10 nm in diameter. Bar: 1μm.

neuronal population after striatal excitotoxic lesion or forebrain hypoxic-ischemic injury in immature rats (Macaya et al., 1994), and during cell loss in Parkinson's disease (Anglade et al., 1997). Interestingly, condensed chromatin has already been detected in melanized neurons stained for in situ detection of DNA fragmentation in the substantia nigra of normal aged subjects (Tompkins and Hill, 1995). However, observation of chromatin condensation at light microscopic level and detection of DNA fragmentation are not unequivocal criteria of apoptosis. We cannot exclude that apoptosis observed in the substantia nigra of subject 2 might have been caused by the cerebral infarction detected in the contralateral hemisphere. However, previous studies reported that hypoxic-ischemic or excitotoxic striatal injury during development did not significantly affect the number of nigral DA neurons and pyknotic cells in adult rats in the contralateral substantia nigra (Burke et al., 1992; Macaya et al., 1994).

The endoplasmic reticulum of dying neurons had normal morphology, as observed in the substantia nigra of patients with Parkinson's disease (Anglade et al., 1997). The darkening and shrinkage of mitochondria detected in all the apoptotic neurons was never significant in non apoptotic neurons, and thus seems to be related to the final step of cell death. This type of mitochondrial alteration was not detected in a group of parkinsonian patients with a mean postmortem delay not statistically different (Anglade et al., 1997). It is possible that the process of cell degradation affects mitochondria later in the pathology, only when identification of DA cell remains becomes uncertain. Alternatively, mitochondrial insult may be one of the events leading to apoptotic cell death during senescence. Contact loss between dying neurons and their surrounding cells was consistently observed in the subjects analyzed in this study. Cell death can be prevented by rescue factors, such as cytokines or cell adhesion molecules (Bellamy et al., 1995), provided through cell-cell or cell-extracellular matrix interactions. Apoptosis occurring in DA neurons during senescence may thus be triggered by deprivation in survival factor, following contact loss from surrounding tissue. Alternatively, a decreased expression of cell adhesion molecules in DA neurons may occur as a consequence of an already ongoing process of cell death. Loss of contact of nigral DA neurons undergoing apoptosis was not detected in a previous study performed in parkinsonian patients (Anglade et al., 1997). Such difference between aging and pathology may be associated with differential expression of factors mediating cell-extracellular matrix interactions. Previous studies indicated that Tumor Necrosis Factor-α (TNF-α) and interferon-γ could modulate the expression of ß3 subunit of the vitronectin receptor, a cell surface protein involved in cell adhesion, in human endothelial cells (Defilippi et al., 1991). Interestingly, TNF-α was detected in glial cells of the substantia nigra only in parkinsonian patients (Boka et al., 1994). The mean age of the senescent subjects (mean±SEM: 82±4.7 years), though not statistically different, was higher than that of parkinsonian patients previously studied (mean±SEM: 75±7 years). Thus, it cannot be excluded that contact loss observed in this study between dying DA neurons and the surrounding tissues may be related to aging.

Since only fragments of melanized neurons, but no complete cells, were detected in glial cells, DA cells may split in membrane-bound apoptotic bodies before being engulfed by glia. This hypothesis is supported by the observation of cell fragmentation in one DA neuron that did not appear to be engulfed in phagocyte. Although the total number of neurons analyzed was too small to draw general conclusions, removal of DA cells dying by apoptosis during aging may be the result of a series of events including separation from adjacent tissue, fragmentation and engulfment of cell corpses in glial cells. This process of cell clearance is commonly found in apoptosis occurring in the renewal of epithelial or parenchymal cells of glandular tissue (Kerr et al., 1972). In contrast, engulfment of a whole apoptotic DA neuron in glial cell was observed in Parkinson's disease (Anglade et al., 1997). This suggests that, contrary to aging, cell absorption in Parkinson's disease may occur rather early in the cell death process. Early phagocytosis in apoptotic process has already been described during the development of *C. elegans*, where engulfment may begin even before the division giving rise to the dying cell is achieved (Driscoll and Chalfie, 1992).

The percentage of apoptotic neurons (2%) observed in this study is lower than that found in the substantia nigra of parkinsonian patients of the same age (6%). These results parallel a previous quantitative estimation in which a greater number of dead cells was reported in the substantia nigra of patients with Parkinson's disease than control subjects, suggesting the presence of pathological process until death (McGeer et al., 1988). The present data indicate that in aging, like in Parkinson's disease, nigral DA neurons die by an active cell death process. However, morphological abnormalities observed in apoptotic neurons during aging, such as chromatin condensed in small patches, mitochondrial alteration and contact loss, were not observed in Parkinson's disease. This suggests that the mechanisms underlying cell death of nigral DA neurons are different in aging and pathology. Since *bcl-2* is expressed in nigral DA neurons of normal aged subjects and patients with Parkinson's disease (Vyas et al., unpublished results), apoptotic process probably involves the neutralization of a protection system against cell death in both cases.

Morphological abnormalities observed at variable degrees in non apoptotic neurons, mitochondrial vacuolation and abnormal filamentous aggregates, suggest that DA neurons may undergo progressive degeneration before final apoptotic cell death. However, these abnormalities might have no direct relation to the process leading to cell death since they were not detected in apoptotic neurons. Interestingly, prominent

mitochondrial vacuolation was not found in a previous ultrastructural analysis of nigral DA neurons in parkinsonian patients, through sparse swollen mitochondria were observed (Anglade et al., 1997). Various alterations of mitochondria associated with aging, such as swelling, abnormal inclusions, increased matrix density, have already been reported in rodents (Johnson et al., 1975; Vanneste and Van den Bosch de Aguilar, 1981) and human (Forno and Norville, 1976; Issidorides and Pappas, 1988). Swelling of mitochondria with damaged cristae and crest disappearance has been observed in neuronal cells when oxidative metabolism was decreased in various in vivo and in vitro models of cell death, such as animals treated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Forno et al., 1988; Rapisardi et al., 1990) and neuroblastoma cells deprived in thiamine (Bettendorf et al., 1995). The accumulations of tightly stacked filaments of 10 nm may correspond to eosinophilic granules previously described in melanized neurons of substantia nigra and locus coeruleus in normal aged brain and Parkinson's disease (Jellinger, 1989). Although the significance of such aggregates is not known, it might reflect an abnormal metabolic pathway related to aging, since the aggregates were more frequent in the oldest subject. However, this must be confirmed by a quantitative analysis performed on a greater number of subjects, since a previous study reported no obvious relation between age and the presence of eosinophilic granules (Schochet et al., 1970). Interestingly, vacuolar degeneration of mitochondria and neurofilamentous accumulations have also been reported in motor neurons of mice with a mutation in Cu/Zn SOD linked with familial amyotrophic lateral sclerosis (Wong et al., 1995). Mitochondrial vacuolation and abnormal filaments found in nigral DA neurons may thus be the consequence of deficits in oxidative phosphorylation, considered to play a crucial role in the process of senescence (Shigenaga et al., 1994).

Autophagic degeneration, which was detected in nigral DA neurons in parkinsonian patients, was not observed in this study. This suggests, in contrast with Parkinson's disease, an absence of autophagic process in DA cells dying during aging. However, it cannot be excluded that the sample of dying neurons examined was too small to detect autophagic degeneration. Necrosis, associated with loss of cell homeostasis, was not observed in this work. It is thus possible that a decrease in energy production until a critical threshold, where the cell can no longer maintain homeostatic functions, is not involved in the demise of DA cells during senescence.

In conclusion, the present data indicate that during aging, nigral DA neurons die through apoptosis, an active process of cell death. The comparison of ultrastructural abnormalities found in dying DA neurons in normal aged subjects and parkinsonian patients suggests that the mechanisms underlying apoptosis are different in aging and Parkinson's disease. Molecules involved in these mechanisms remain to be identified. Morphological signs of oxidative metabolism dysfunction was observed in a significant number of DA neurons in the normal aged subjects. Whether oxidative stress is directly involved in the process leading to apoptosis during normal aging is still an enigma.

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