Histol Histopathol (2006) 21: 361-366

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Apolipoprotein D expression in substantia nigra of Parkinson disease

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Summary. Apolipoprotein D (apo D), a lipocalin transporter of small hydrophobic molecules could play an important role in several neurodegenerative diseases. However, its role in those diseases remains unclear. Increments of apo D have been reported in relation with injury and degeneration in the nervous system. Recently increases of apo D level have been reported in schizophrenia, a neuropathologic disease where the oxidative stress and lipid abnormalities may be involved. Apo D could act as a sequestering molecule binding excess of arachidonic acid in cells. In order to determine the relationship between apo D expression and other neurodegenerative pathologies related to oxidative damage, we studied the presence of apo D in the substantia nigra of control and Parkinson disease (PD) subjects. We found dopaminergic neurons were not immunoreactive for apo D, control or PD subjects. However, surrounding glial cells showed immunostaining for apo D and signal increases in PD cases. These findings support the role of apolipoprotein D in neuroprotection and the importance of glia in the amount of this protein in the central nervous system.

Key words: Apo D, Dopaminergic neurons, Glial cells, Immunohistochemistry, Neuromelanin

Introduction

Parkinson disease (PD) is a neurodegenerative disorder characterized by the loss of neuromelanin-containing dopaminergic neurons in the *pars compacta* of the substantia nigra (SN) and by intraneuronal cytoplasmic inclusions called Lewy bodies (Lang and Lozano, 1988a,b). PD is a complex disorder with many different causes, yet studies seem to find common pathways (Dawson and Dawson, 2003). Oxidative stress is believed to contribute to degeneration of dopaminergic neurons by means of a neurotoxic mechanism mediated by free radicals. Several specific biochemical features can explain the vulnerability of these neurons to oxidative stress (Fahn and Cohen, 1992).The oxidative metabolism of dopamine has the potential to generate cytotoxic free radicals. Dopamine can be oxidized by either monoamine oxidase or undergo autooxidation to generate hydrogen peroxide. This molecule can damage the neuron directly or indirectly through the formation of hydroxyl radicals in the presence of ferrous ions. Neuromelanin present within the SN neurons has the potential role to promote site-specific accumulation and reduction of iron, thereby potentiating iron-induced lipid peroxidation and consequent cell death (Youdim and Riederer, 1997). Glutathione (GSH), superoxide dismutase (SOD) and catalase are the most important enzymes of the cell antioxidant defense system. Several findings of decreased antioxidant defense mechanisms are reduced in parkinsonian SN. GSH is depleted in PD and it has been suggested that this loss may be an early event in the pathogenesis (Dexter et al., 1994). Inhibition of GSH synthesis causes neuronal death in primary cell cultures and cell lines. It has also been described that the up-regulation of SOD activity may provide protection from oxidative stress when the depletion of GSH occurs (Jenner and Olanow, 1996). Inhibition of lipoxygenase activity prevents cell death, implicating the arachidonic acid (AA) metabolism in the toxicity of GSH depletion (Li et al., 1997; Mytilineou et al., 1999). The metabolism of AA via the lipoxygenase pathway results in the generation of oxygen free radicals, which contribute to cellular damage and death. Some studies have suggested that limiting AA release and metabolism may provide benefit in conditions with a depletion of GSH, such as PD (Kramer et al., 2004).

Apolipoprotein D (apo D), a member of the lipocalin family of proteins, binds to AA and cholesterol among other hydrophobic molecules (Morais-Cabral et al., 1995; Rassart et al., 2000). In addition to the abundant expression in human serum and many tissues, apo D is also widely expressed in the brain (Drayna et al., 1986; Navarro et al., 1998). Furthermore, apo D expression increases in the brain during development and aging

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(Kalman et al., 2000; Sánchez et al., 2002), as well as after degeneration and regeneration processes (Spreyer et al., 1990; Ong et al., 1997; Terrisse et al., 1999). Elevated apo D levels have also been reported in brains affected by some neurodegenerative diseases like Niemann-Pick (NPD), Alzheimer (AD), and schizophrenia. With respect to the up-regulation of apo D levels, some authors suggest that apo D may be elevated in human brains in response to local pathophysiology (Suresh et al., 1998; Terrisse et al., 1998; Thomas et al., 2003a). Apo D is thought to act as a response protein and increased levels of apo D may be a natural response to neuronal damage (del Valle et al., 2001). A growing body of evidence implicates free radical toxicity, oxidative enzyme impairment and mitochondrial dysfunction in neurodegenerative diseases (Rao and Balachandran, 2002). The elevated levels of apo D in post-mortem brains, as well as plasma, added to new evidence for an excess of free radical formation and lipid peroxidation in schizophrenia, suggest that apo D may act to stabilize membrane-associated AA by preventing release and chaperoning free AA in the cell (Thomas et al., 2001, 2003a,b; Khan et al., 2003).

Together, those lines of evidence suggest that dopaminergic neurons in PD would express apo D either as a neurodegenerative marker or as a chaperon molecule which protects against free radical damage. According to this hypothesis, we investigated by means of immunocytochemistry the presence of apo D in dopaminergic neurons of SN from normal and Parkinson subjects to determine if there were disease-specific changes in apo D expression in this stressed neuronal population.

Material and methods

Twelve human brains, from 6 patients (2 females, 4 males, aged 70.2±10) with PD and 6 (3 females, 3 males, aged 75.1±7.2) control, provided by the Pathology Department of The Central Hospital of Asturias, were used in the present study. PD patients had been clinically diagnosed and the pathology confirmed post-mortem. All the patients with PD showed nigral degeneration and Lewy bodies in the remaining neurons. None of the control subjects had nigral or strial lesions. The pieces from midbrain at the colliculus level were obtained from necropsy, and fixed by immersion in 10% buffered formalin. After fixation, the blocks were washed in distilled water, dehydrated through successive alcohols, cleared in two baths of butyl acetate, embedded in paraffin and blocked out in a suitable mold. Transverse sections about 10 µm thick were obtained and attached to gelatine-covered slides.

Sections were deparaffined in xylene and rehydrated, and an immunocytochemical method was carried out for detection of apo D or α-synuclein. The sections were treated sequentially with Triton X (0.1%, five minutes) at room temperature, washed in distilled water, treated with H_2O_2 (3%, five minutes) in a wet chamber at room temperature, washed in distilled water and treated with phosphate-buffered saline(PBS) (two minutes). Non-specific binding was blocked by incubation with bovine serum (30 minutes at room temperature, in a wet chamber). Incubation with a specific antibody against human apo D (1:2000 dilution; antibody was a gift from Dr. Carlos López-Otín, Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo (Díez-Itza et al., 1994; Navarro et al., 1998)) or α-synuclein (1:2000 dilution, Chemicon, AB 5038) was carried out overnight at 4°C. After several washes in PBS the sections were incubated for 30 minutes at room temperature using a biotinylated horse universal antibody (Vector, PK-8800) diluted 1:50. The sections immunostained for apo D were subsequently incubated with streptavidin labelled with "Fluorolink Cy2" (Amersham, PA-42001) and mounted in aqueous mounting media. These sections were not counterstained.

Those samples immunostained for α-synuclein, were incubated with Extravidin (Sigma Extra-3) labelled with HRP. The peroxidase activity was visualized in a red AEC reaction (Sigma, A-6926) [0.5 mg AEC, 50 µl dimetylformamide, 10 µl H^2O^2 (3%) in 940 µl acetate buffer]. Finally, sections were counterstained with a modification of the formaldehyde-thionin technique (Tolivia et al., 1994) and were mounted in aqueous mounting media. Sections were photographed with a digital Nikon camera (DN 100) in a Nikon Eclipse E400 microscope.

The habitual specificity control tests were carried out. Control sections were incubated with buffer for antibody dilution or apo D antibody, preabsorbed with apo D immunizing peptide (80 µg/ml). This showed an absence of staining.

The immunocytochemical signal was selected with Photoshop (Lehr et al., 1997) and quantified with NIH Image 1.57 software. The data obtained from the densitometric study were subjected to one-way analysis of variance (ANOVA) to discern differences between controls and PD subjects, and then Student's t test (two tailed) was used to determine exact p values (to evaluate differences between both). A $p \leq 0.05$ was considered as significant. Statistical analysis was performed using SPSS 6.01 for Windows.

Results

The cytoarchitectonic study of control subjects showed, as expected, several pigmented neurons in SN. These neurons showed different shapes but are characterized by the presence of a large amount of neuromelanin, which frequently filled the neuronal soma (Fig. 1A). The number of neurons and the pigment appears to decrease with age. The study of immunostained sections of SN shows no signal for apo D in neurons. Apo D positive signal only appeared in the surrounding glial cells (Fig. 1C). The immunoreactivity and the number of positive glial cells were apparently

incremented with age. In contrast to this region, other midbrain nuclei such as oculomotor nucleus, superior colliculus and midbrain raphe, showed immunoreactivity in their neurons.

The study of PD patients showed a moderate to severe loss of dopaminergic neurons of SN. Many neurons presented a degradation of the intracellular pigment and frequently it was found in the neuropil as a consequence of neuronal death (Fig. 1B). The immunocytochemical study for α-synuclein confirmed the presence of Lewy bodies in a large number of neurons (Fig. 1E). Neither remaining nor degenerated nigral neurons showed immunoreactivity for apo D. As it occurs in control patients, the surrounding glia was apo D positive. The number of glial cells was clearly incremented with respect to controls and showed an increased in the intensity of the signal (Fig. 1D). The densitometric analysis of the immunocytochemical signal showed that the relative density of apo D was significantly higher in PD (Fig. 2). Other nuclei on the midbrain section, as it occurs in control subjects, showed high levels of apo D immunopositivity in both glial and neuronal cells (Fig. 1F) that apparently increase with age.

The overall impression of the present study is that neurons of SN cannot express apo D in normal and in degeneration conditions. However, glial cells have an important expression for apo D that increases in PD but the neurons of SN, in contrast to other neuronal nuclei, cannot obtain apo D from the surrounding glia.

Discussion

The immunocytochemical study for apo D in the

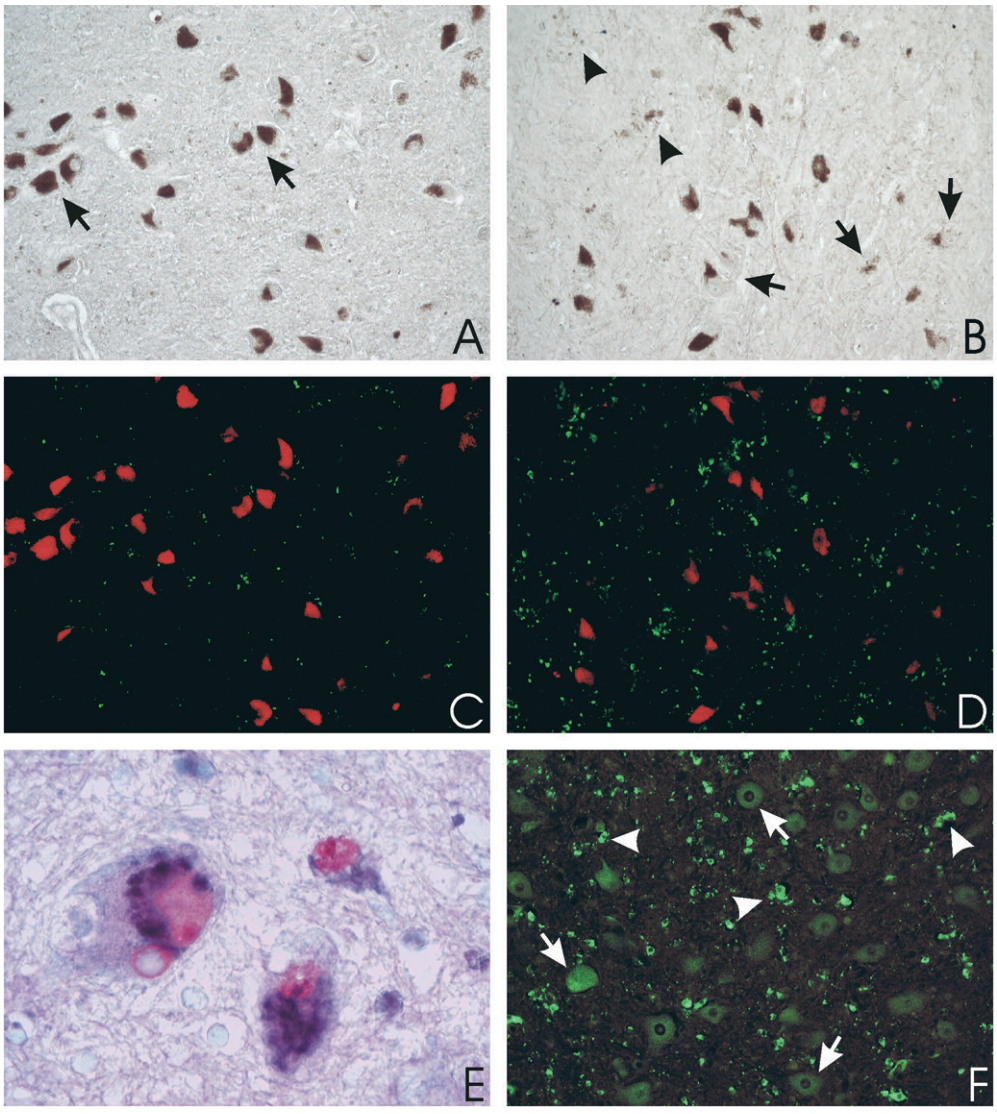

Fig. 1. A. No-stained substantia nigra of subject control. The characteristic presence of neuromelanine in the neurons as a brown deposit is observed (arrows). **B.** Non stained substantia nigra of Parkinson's disease patient. The loss of neuromelanine in neurons (arrowheads) and its presence in the neuropil (arrows) is clearly observed. **C.** This microphotograph shows the superposition of Fig. 1A and its immunofluorescence image with apo D. The neuromelanine containing neurons (red) do not show presence of apo D (green). **D.** Superposition of fig. 1B and its immunofluorescence image with apo D. The presence of apo D is clearly incremented (green) but the neurons (red) do not show immunostaining for apo D. **E.** Image of SN neurons of PD patient immunostained for α-synuclein. The presence of positive Lewy bodies is observed (red). **F.** Red nucleus of Parkinson's disease patient. The presence of apo D is clearly observed in both glial (arrows) and neuronal cells (arrowheads). A-D,F, x 160; E, x 800

substantia nigra of PD and control subjects showed the absence of positive neurons in this region. In contrast, glial cells were positive for apo D in both cases, but there is a higher intensity of immunostaining in PD. Meanwhile, other midbrain nuclei presented immunoreactivity for apo D in neurons and glial cells. The intensity of the signal was apparently incremented with age.

Apo D has been related with several pathological conditions in the nervous system. In the peripheral nervous system, high levels of apo D are present in the regenerating nerve where it could be involved in lipid transfer (Boyles et al., 1990). The general notion, in the central nervous system, is that affected regions have stronger levels of apo D than controls. A role for apo D in neuronal degeneration has been suggested in kainic acid-induced excitotoxic injury of rat brain, wherein it has been suggested a specific induction of apo D in hippocampal pyramidal neurons destined for cell death (Ong et al., 1997). Similar results for apo D have been obtained in the cortex, following unilateral cortical impact injury, but not in hippocampus where the injury did not result in overt necrosis or apoptosis cell death (Franz et al., 1999). These authors suggest the possibility that apo D may have a specific role in neuroprotection and regeneration.

Several studies have been reported on apo D protein and mRNA levels in brains affected by neurodegenerative disease. Apo D protein levels were found to be higher in hippocampus and cerebrospinal fluid (CSF) but not in frontal or temporal cortices of AD patients (Suresh et al., 1998; Kalman et al., 2000). By immunocytochemistry, however, apo D has been found in the senile plaques (Navarro et al., 2001) and higher percentages of apo D in reactive neurons (Kalman et al., 2000; Belloir et al., 2001). In addition, the percentage of apo D immunoreactive neurons correlated with the number of neurofibrillary tangles (NFT) but not with the number of senile plaques (Belloir et al., 2001; Glöckner and Ohm, 2003). The hypothesis that apo D gene expression is increased in stressed cortical neurons before they accumulate NFT has been suggested (Belloir et al., 2001). Within this context, an increased amount of apo D is observed in the NPD-C mouse cerebellum linked to a selective degeneration of Purkinje neurons (Suresh et al., 1998). Recent studies on distribution of apo E and apo D in cerebral ß-amyloid deposits suggest that apo D could play a role opposite to that of apo E in ß-amyloid deposition and fibril formation (Navarro et al., 2003). These results suggest a possible role of apo D in cellular response to a progressive injury which could act as a factor preserving cellular function from cellular degeneration in senile plaques and amyloid-containing vessels.

Recently, an apo D increase was reported in plasma and certain brain regions of schizophrenic and bipolar patients (Thomas et al., 2003a). These results summed up the evidence for an increased oxidative stress in schizophrenia, suggesting that apo D may have a role in the pathophysiology of the illness (Thomas et al., 2001b; Khan et al., 2003). The phospholipid hypothesis of schizophrenia proposes an increased rate of removal of essential fatty acids, especially AA and docosahexaenoic acid (DHA), from the membrane coupled with a reduced rate of incorporation (Horrobin, 1998). AA can be converted into a variety of biologically active compounds, such as eicosanoids, which serve as potent messengers in regulating the inflammatory response that may affect schizophrenic psychopathology. Atypical antipsychotics increase the expression of antioxidant enzymes that combined with the increment of trophic factors is a common mechanism related with neuroprotection (Parikh et al., 2003). The up-regulated expression of apo D by atypical antipsychotics, such as clozapine (CLOZ), may act to stabilize membrane-associated AA by preventing release and chaperoning free AA in the cell (Thomas et al., 2001a; Khan et al., 2003). In this hypothesis, apo D may be a neuroprotective molecule as has been previously proposed (Franz et al., 1999; del Valle et al., 2001).

Oxidative damage also occurs in the brain in PD, as shown by an increased in lipid peroxidation and DNA damage in the substantia nigra (Jenner and Olanow, 1998). The suggested protective function of apo D may be important for the survival of dopaminergic neurons, since they are constantly threatened by oxyradicals produced during dopamine oxidation. A link has been reported between an overproduction of arichinodate and an increase of apo D expression in rat hippocampus after an excytotoxic injury (Montpied et al., 1999). As in schizophrenia, alterations in membrane phospholipids have been reported in PD (for review see Horrobin and Benneth, 1999). The generation of superoxide radicals

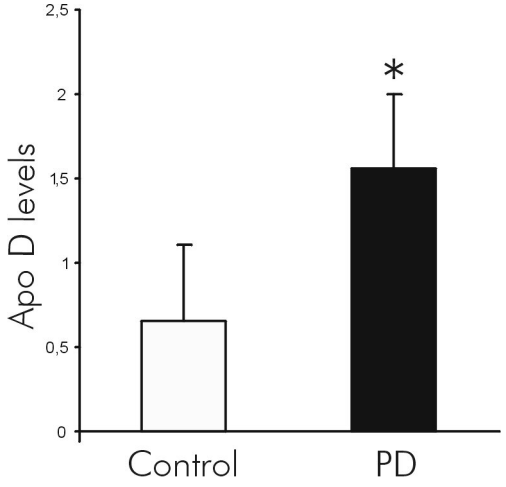

Fig. 2. Densitometric analysis of apo D signal in glial cells of controls and PD subjects. A significant increase in apo D expression is observed in PD patients. Asterisk indicates significant differences.

during the metabolism of AA is likely to play an important role in the toxic events that follow GSH depletion in dopaminergic neurons (Mytilineou et al., 2002). However, we did not find immunoreactive neurons for apo D in substantia nigra either in controls or in PD. The lack of apo D induction that we observed in these neurons supports the regional specificity for the changes in apo D expression (Thomas et al., 2003a). Therefore, we conclude that these nigral neurons cannot express apo D in response to oxidative stress.

We found that the glia of the nucleus presents apo D expression in control and in PD subjects. Astrocytes and oligodendrocytes localize and synthesize apo D in normal nervous system as has been described (Patel et al., 1995; Navarro et al., 1998, 2004). However, the number and intensity of signal for apo D immunoreactive glial cells are increased in PD. These results are in agreement with previous works where it has been demonstrated that reactive astrocytes overexpress apo D (Trieu and Uckum, 2000; del Valle et al., 2003). The apo D in reactive astrocytes probably acts as a lipid transporter protein in neurite promoting responses, and apo D might be involved in repair and regeneration or in removal of neurotoxic molecules after cell death (del Valle et al., 2003). The loss of neurons in substantia nigra is associated with a glial response composed mainly of activated microglial cells and reactive astrocytes, which explain the increase of apo D in surrounding glia. The glial response may be the source of trophic factors and can protect neurons against reactive oxygen species and GSH (Makar et al., 1994). During conditions of oxidative stress, the state of the surrounding glia can determine whether neurons will survive or die. Some studies have proved that astrocytes, at least partially, could delay neuronal death in pathological situations in which a peroxide radical has been involved (Jenner and Olanow, 1998; Desagher et al., 1996). In this regard, it raises the possibility that there is an impairment of apo D traffic between glia and neurons in substantia nigra in PD which may affect neuron survival.

In summary, our data support that apo D may have a different regulation in brain regions, and its expression may not necessarily be linked with neuronal lesion or death. According to other previous reports, apo D could be a region marker for pathological processes and its accumulation might be potentially beneficial for cells. The fact that nigral dopaminergic neurons could not express or obtain apo D from the surrounding glia, and the vulnerability of these cells to stressors, allow us to think that apo D plays a significant role in neuronal protection as has been previously proposed (del Valle et al., 2001, 2003; Franz et al., 1999; Navarro et al., 1998, 2004). The possibility to control the apo D up-regulation in these neurons by pharmacological products (or in the future the modification of stem cells, which could be transplanted, to express high levels of apo D) would be considered as a possible strategy to prevent neurodegenerative effects of oxidative stress in PD.

Acknowledgements. This work was supported by grants from Fondo de Investigación Sanitaria Española (02- PI020324 and 03-RED-C03/06).

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Accepted October 11, 2005