ACTA STEREOL 1996; 15/2: 141-146
ORIGINAL SCIENTIFIC PAPER

A MORPHOMETRIC STUDY FOR CYTOCHROME C OXIDASE OF THE SUBSTANTIA NIGRA IN NORMAL AGING AND PARKINSON's DISEASE

Kyoko Itoh, Josef Müller-Höcker*, Parviz Mehraein, Serge Weis

Institute of Neuropathology and *Institute of Pathology, Ludwig-Maximilians-University, D-80337 Munich, Germany

ABSTRACT

Degeneration of the dopaminergic nigrostriatal system has been reported to occur with physiological aging as well as in Parkinson's disease (PD). Defects of respiratory chain complexes were interpreted as a possible pathogenetic mechanism for cell death.

In the present study, the substantia nigra of brains with normal aging and of brains from patients with PD was investigated by means of morphometry and immunohistochemistry. The antero-medial (**Am**), antero-intermediolateral (**Ail**), postero-medial (**Pm**), and postero-lateral (**Pl**) nuclei of the substantia nigra were analysed using antibodies directed against the subunits II/III of cytochrome c oxidase (COX), the complex IV of the respiratory chain.

In normal aging, the numerical density of melanin-positive neurons with COX defects was significantly increased in the four investigated nuclei, namely **Am**, **Ail**, **Pm**, and **Pl**. The total number of neurons was only significantly decreased in the *Pm* nucleus. Intact neurons without COX defects were significantly reduced in the **Pm** and the **Pl** nuclei. The size of all types of neurons showed no significant differences with aging. In Parkinson's disease, the numerical density of pigmented neurons with COX defects were significantly increased only in the **Pl** nucleus, whereas pigmented neurons without COX defects was significantly reduced in the nuclei **Am**, **Ail** and **Pl.** The cell size of pigmented neurons without COX defects was significantly reduced in the nuclei **Am** and **Pl** of PD cases.

The data of the present study indicate that complex IV defects of neurons in the substantia nigra might be one cause of neuronal dysfunction occuring during physiologic aging. Furthermore, it is suggested that complex IV defects in nigral neurons are most probably due to accelerated aging rather than being a primary pathogenetic mechanism of PD.

Key words: Cytochrome c oxidase, normal aging, morphometry, Parkinson's disease, substantia nigra.

INTRODUCTION

Loss of neurons in the nigrostriatal dopaminergic system with physiologic aging has been described both in rats and in humans (Fearnley et al., 1991). Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons in the substantia nigra (SN) and other brainstem nuclei (Fearnley et al., 1991; Gibb et al., 1991). It was suggested that the deterioration of the dopaminergic nigrostriatal system with age resulted from autodestruction

due to the accumulation of reactive oxygen intermediates which were derived from oxidative metabolites of dopamine (Felton et al., 1992). Recently, it was suggested that defects of mitochondrial respiratory chain complexes might play a crucial role in the pathogenesis of neuronal death (Benecke et al., 1993; Cardellach et al., 1993; DiMauro, 1993; Hattori et al., 1991; Mann et al., 1992; Parker et al., 1989; Schapira et al., 1989; Schapira et al., 1994; Shoffner et al., 1991). Until now, there exist only a few immunohistochemical studies demonstrating defects of mitochondrial enzymes in the SN of PD cases. Hattori et al. (1991) described that immunoreactivity of complex I but not of the complexes II, III, and IV was significantly reduced in the pigmented neurons of the substantia nigra in PD patients.

In the present study, immunohistochemical and morphometric assessments of the substantia nigra in normal aging brains and in brains from PD cases were performed in order to detect at the cellular level defects in the respiratory chain complex IV (COX).

MATERIAL AND METHODS

The substantia nigra of 36 human brains from persons without evidence of central nervous system involvement during life and without neuropathological changes was examined by means of immunohistochemistry and morphometry. The age of the investigated brains ranged from 35 to 98 years. The substantia nigra of 8 patients with PD as well as of 10 age-matched controls without neuropathological changes was also examined. The average age was 74.7 years (age range: 68-81 years) in PD. After formalin-fixation, the specimens for histopathological examination were taken from the midbrain at the level of the oculomotor nucleus and the caudal portion of the red nucleus. Immunohistochemistry was performed on paraffin-embedded sections following standard protocols. The ABC method was applied and AEC was used to visualize the reaction product as previously described (Müller-Höcker, 1992). The antibody, kindly provided by Prof. Dr. B. Kadenbach, Department of Biochemistry, University of Marburg/Lahn, Germany, was used at a dilution of 1:1000.

For the morphometric evaluation of the substantia nigra, pars compacta, the following nigral nuclei were selected after clear identification on histological sections: (a) antero-medial: **Am**, (b) antero-intermediolateral: **Ail**, (c) postero-medial: **Pm**, (d) postero-lateral: **Pl**. The morphometric evaluation of the different nuclei in the substantia nigra was done as follows. A measuring field (15 x 20 cm) was overlaid on the histological section on the TV monitor. The neurons were analysed at a magnification of x1429. The numerical density of neurons within a nucleus was calculated as the number of cells per square millimeter (n/mm^2) and was determined following the rule of the unbiased test grid (Gundersen, 1977). The size of neurons was determined using the point counting method and was expressed in μm^2.

The numerical density and the size of the following types of neurons were determined: (a) pigmented neurons without COX defects, i.e. neurons containing melanin with immunoreactivity for the subunit II/III of COX (*MpCp*), (b) pigmented neurons with COX defects (*MpCn*), (c) non-pigmented neurons without COX defects (*MnCp*), and (d) non-pigmented neurons with COX defects (*MnCn*). At least fifteen fields per nucleus of the SN were counted following the random systematic sampling (Weis, 1991). Correlation, regression analysis, one-way analysis of variance (ANOVA), and the non-parametric Mann-Whitney test were used.

RESULTS

Normal Aging

The ANOVA analysis showed that the parameters 'age' and 'nucleus' had a significant effect upon the numerical density. There was no significant difference between the left and the right side (data not shown). The numerical density of pigmented neurons with COX defects (*MpCn*) was significantly increased with age. The numerical density of all neurons (*Tot*) was significantly reduced and that of pigmented neurons without COX defects (*MpCp*) was significantly reduced with age. When analysing the different nuclei separately, the numerical density of pigmented neurons with COX defects (*MpCn*) was significantly increased in all investigated nuclei **Am**, **Ail**, **Pm** and **Pl** with age. The numerical density of all neurons (*Tot*) was significantly reduced only in the nucleus **Pm**, whereas the numerical density of pigmented neurons without COX defects (*MpCp*) was significantly reduced in the nuclei **Pm** and **Pl** with advancing age (Table 1). Non-pigmented neurons with COX defects (*MnCn*) were not found.

Table 1. Correlation coefficients for the numerical density of the various cell types in the different nuclei with age. (: $p < .01$ and **: $p < .001$)*

	all nucl.	Am	Ail	Pl	Pm
MpCp	- .45 **	- .31	- .19	- .48 **	- .53 **
MnCp	.17	.08	.06	.14	.31
MpCn	.68 **	.72 **	.72	.76 **	.62 **
MnCn	.	.	.	.	.
Tot	- .23 *	- .19	.09	- .18	- .35 *

The size of neurons differed among the analysed cell types and among the different nuclei; however, no significant differences in size with advancing age could be described (data not shown).

Parkinson's disease

The numerical density of pigmented neurons with COX defects (*MpCn*) was significantly increased in PD as compard to control brains. The numerical density of all neurons (*Tot*) was significantly reduced in PD and that of pigmented neurons without COX defects (*MpCp*) was significantly reduced in PD as compared to controls. The numerical density of non-pigmented neurons without COX defects (*MnCp*) was significantly increased in PD as compared to control brains. When analysing the different nuclei separately, the numerical density of pigmented neurons with COX defects (*MpCn*) was significantly increased only in the nucleus **Pl** (Fig.1). The numerical density of all neurons (*Tot*) was significantly reduced in all investigated nuclei (**Am, Ail, Pl** and **Pm**) of PD cases and that of pigmented neurons without COX defects (*MpCp*) was significantly reduced in the nuclei **Am, Ail** and **Pl** of PD cases (Fig.1). The numerical density of non-pigmented neurons without COX defects (*MnCp*) was significantly increased in the nuclei **Am, Ail**, and **Pl** of PD cases. Non-pigmented neurons with COX defects (*MnCn*) were not found.

The cell size of all neurons (*Tot*) was significantly reduced in the nuclei **Am**, **Pm** and **Pl** of PD cases as compared to control brains. The cell size of pigmented neurons without COX defects

(*MpCp*) was significantly reduced in **Am** and **Pl** of PD cases as compared to control brains. The cell size of *MpCn* and of *MnCp* did not differ significantly between PD cases and normal controls.

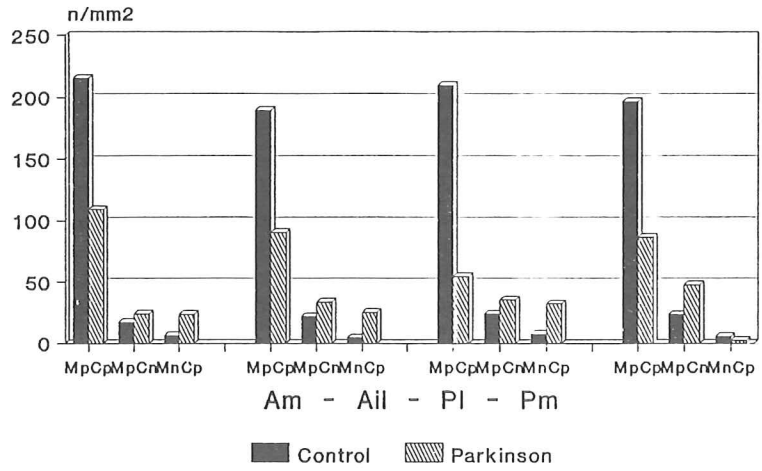

Fig. 1. Numerical density (n/mm²) of the evaluated cell types in the different nuclei of the substania nigra in normal controls and in PD.

DISCUSSION

Based on morphological analysis, Müller-Höcker et al. (1989) reported that the loss of cytochrome c oxidase appears to be a general aging phenomenon, with organ dependent manifestation rates, indicating different organ and cellular sensitivity to the aging process. Since the first report by Shapira et al. (1989) showing a defect of the mitochondrial respiratory chain complex I in the SN of nine PD cases, these findings have been confirmed by later investigators (Mann et al., 1992; Schapira, 1994). It was suggested that changes seen in PD could be attributed to a multisystem decline of mitochondrial respiratory chain complexes. Several investigators showed that these defects could be found not only in the SN but also in other brain regions, muscle tissue, and platelets (Benecke et al., 1993; Cardellach et al., 1993; Parker et al., 1989; Shoffner et al., 1991). However, these results could not consistently be replicated by various research groups (Mann et al., 1992; Schapira, 1994).

The defects of the mitochondrial respiratory chain complexes are thought to be due to specific deletions of mitochondrial DNA (mtDNA) (Ikebe et al., 1990; Ozawa et al., 1990). The "common deletion" was significantly increased in the brain with age, especially in brain structures characterized by a high dopamine metabolism (putamen, caudate nucleus and substantia nigra) (Corral-Debrinski et al., 1992). There is a progressive age-related accumulation in oxidative damage to DNA and the mtDNA is 10-fold preferentially affected (Ozawa et al., 1990). Ikebe et al. (1990) reported that the common deletion was found in a significantly higher number in the striatum of 5 PD cases than in age-matched controls. Ozawa et al. (1990) reported that the proportion of mutant mtDNA as related to normal mtDNA was at least ten times higher in the striatum of PD cases than

in normal controls. In contrast, considerable mutations of mtDNA in the SN and muscle tissue of PD cases could not be detected by several research groups (Mann et al., 1992; Schapira, 1994; Shoffner et al., 1991).

This is the first report of a combined immunohistochemical and morphometric study showing defects of complex IV in various nuclei of the SN in PD cases as well as in normal control brains. In the present retrospective study, numerical densities of nerve cells were determined while absolute numbers could not be calculated. Complex IV defects were detected only in pigmented neurons of the SN both in PD and normal controls. Thus, melanin-containing neurons were selectively damaged in their mitochondrial function.

In normal aging, the numerical density of pigmented neurons with COX defects was significantly increased in all investigated nuclei, however, that of total neurons was significantly reduced in the nuclei **Pm**. This suggests that COX defects might be fairly responsible for a neuronal loss or cause a cell dysfunction prior to neuronal death. The fact that non-pigmented neurons without COX defects increased in the nuclei **Pm** suggests that neurons without neuromelanin were relatively preserved and resistant against this phenomenon or that these neurons lost their melanin accompanied by functional deficits; if so, the latter possibly constitutes another pathway leading to cell death.

In PD, the numerical density of pigmented neurons with COX defects was significantly increased only in the nucleus **Pl**, whereas, the number of all neurons was significantly reduced in the four investigated nuclei. In addition to neuronal dropout, the cell size of the pigmented neurons without and with COX defects was significantly reduced in the nuclei **Am** and **Pl** of PD cases. The data suggest that COX defects are most probably due to cell aging rather than being directly involved in the pathogenesis of PD.

The relationship between complex IV defects and neuronal loss is still unclear. The selective vulnerability of cathecholaminergic neurons has been described in the SN of PD cases and of aging brains (Fearnley et al., 1991; Gibb et al., 1991; Hirsch, 1992). Cathecholaminergic neurons contain neuromelanin, which is produced through an autooxidative metabolism leading to the production of oxygen free radicals, and thus are physiologically exposed to more oxidative stress. The defects of various complexes of the respiratory chain, including complex IV, might also cause an increased formation of free radicals. These free radicals show cell toxicity for melanin containing neurons. The oxidative phosphorylation defects result in a decrease of ATP production leading to energy impairment, which is also responsible for neuronal death (DiMauro, 1993; Schapira, 1994).

In conclusion, complex IV defects of pigmented neurons in the substantia nigra pars compacta increase with aging; these COX defects might be responsible for physiological alterations of cell function leading to neuronal death in elderly persons. The complex IV defects in PD are least likely to represent one of the primary pathogenetic factors which induce neuronal death. Further investigations should be performed in order to investigate the role of the co-existing factors and to elucidate the degenerative process at work by combining morphologic, immunohistochemical and molecular biologic data.

ACKNOWLEDGEMENTS

The authors thank Ms Barbara Germann, Petra Meier, and Sabine Schäfer for their skillful technical assistance. The help of Ida C. Llenos, MD, in correcting the manuscript is highly appreciated. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Mu 755/1-3).

REFERENCES

Benecke R, Strümper P, Weiss H. Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson's-plus syndromes. Brain 1993; 116: 1451-1463.

Cardellach C, Marti MJ, Fernandez-Sola J et al. Mitochondrial respiratory chain activity in skeletal muscle from patients with Parkinson's disease. Neurology 1993; 43: 2258-2262.

Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF, Wallace DC. Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nature Genetics 1992; 2: 324-329.

DiMauro S. Mitochondrial involvement in Parkinson's disease: the controversy continues. Neurology 1993; 43: 2170-2172.

Fearnley JM, Lees AJ. Aging and Parkinson's disease: substantia nigra regional selectivity. Brain 1991; 114: 2283-2301.

Felton DL, Felton SY, Steece-Collier K, Date I, Clemens JA. Age-related decline in the dopaminergic nigrostriatal system: the oxidative hypothesis and protective strategies. Ann Neurol 1992; 32: S132-S133.

Gibb WRG., Lees AJ. Anatony, pigmentation, ventral and dorsal subpopulations of the substantia nigra, and differential cell death in Parkinson's disease. J Neurol Neurosurg Psychiatry 1991; 54: 388-396.

Gundersen HJG. Notes on the estimation of the numerical density of arbitrary profiles: the edge effects. J Microcopy 1977; 111: 219-223.

Hattori Y, Tanaka M, Ozawa T, Mizuno Y. Immunohistochemical studies on complexes I,II,III, and IV of mitochondria in Parkinson's disease. Ann Neurol 1991; 30: 563-571.

Hirsch EC. Why are nigral catecholaminergic neurons more vulnerable than other cells in Parkinson's disease? Ann Neurol 1992; 32: S88-S93.

Ikebe S, Tanaka M, Ohno K et al. Increase of deleted mtDNA in the striatum in PD and senescence. Biochem Biophys Res Commun 1990; 170: 1044-1048.

Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AHV, Marsden CD. Brain, skeletal muscle, and platelet homogenate mitochondrial function in Parkinson's disease. Brain 1992; 115: 333-342.

Mann VM, Cooper JM, Schapira AHV. Quantitation of a mitochondrial DNA deletion in Parkinson's disease. FEBS Lett 1992; 299: 218-222.

Müller-Höcker J. Cytochrome-c-oxidase deficient cardiomyocytes in the human heart- an age-related phenomenon. Am J Pathol 1989; 134: 1167-1173.

Müller-Höcker J, Seibel P, Schneiderbanger K et al. In situ hybridization of mitochondrial DNA in the heart of a patient with Kearns-Sayre syndrome and dilative cardiomyopathy. Hum Pathol 1992; 23: 1431-1437.

Ozawa T, Tanaka M, Ikebe S, Ohno K, Kondo T, Mizuno Y. Quantitative determination of deleted mitochondrial DNAs relative to normal DNA in parkinsonian striatum by a kinetic PCR analysis. Biochem Biophys Res Commun 1990; 172: 483-489.

Parker WD, Boyson SJ, Parks JK. Abnormalities of electron transport chain in idiopathic Parkinson's disease. Ann Neurol 1989; 26: 719-723.

Schapira AHV, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1989; 1: 1269.

Schapira AHV. Evidence for mitochondrial dysfunction in Parkinson's disease - a critical appraisal. Mov Disord 1994; 1: 125-138.

Shoffner JM, Watts RL, Juncos JL, Torroni A, Wallace DC. Mitochondrial oxidative phosphorylation defects in Parkinson's disease. Ann Neurol 1991; 30: 332-339.

Weis S. Morphometry in the neurosciences. In: Wenger E, Dimitrov L, eds. Digital Image Processing and Computer Graphics. München, Wien: Oldenburg, 1991: 306-325.

Presented at the 9th International Congress for Stereology, Copenhagen, August 20th to 25th, 1995.