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Upregulation of the Anti-apoptotic Protein Bcl-2 May Be an Early Event in Neurodegeneration: Studies on Parkinson's and Incidental Lewy Body Disease

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Apoptosis and oxidative stress have been suggested to be involved in Parkinson's disease (PD). However, whether this is a cause or consequence of neurodegeneration is unknown. Incidental Lewy Body disease (ILBD) appears to be a presymptomatic form of Parkinson's disease where individuals are neurologically normal, but after post-mortem examination pathology similar to Parkinson's disease is present. Thus, ILBD can be used to examine the early stages of the pathological process in PD. We investigated the levels of Bcl-2, an anti-apoptotic protein known to decrease cell death induced by several mechanisms, including oxidative stress. Our data show that Bcl-2 is significantly raised in the basal ganglia regions of PD patients as compared to age-matched controls. A similar trend is also found in ILBD. We propose that Bcl-2 increases in some brain regions as an early event and that these brain regions are under a stress for perhaps many years before any symptomatic changes occur.

Key Words: **Bcl-2, Parkinson's disease, Incidental Lewy Body disease, Oxidative stress.**

Parkinson's disease (PD) results primarily from the degeneration of dopaminergic neurones in the substantia nigra pars compacta, leading to motor impairments. The cause of nigral neuronal loss is unknown but oxidative stress may be involved either as a cause or a consequence of this pathological process (reviewed by (1,2)). Dopamine itself can oxidize to produce reactive oxygen species (ROS) and it can be metabolised by monoamine oxidase B to produce H_2O_2. H_2O_2 is normally metabolised by glutathione peroxidase in the brain but can also react with transition metals such as iron to produce the highly toxic hydroxyl radical (3,4). Hence, treatment of Parkinson's disease with L-DOPA could conceivably contribute to oxidative stress. Studies on post-mortem PD brain tissue have shown increased levels of iron, a decrease in reduced glutathione (GSH) levels, inhibition of mitochondrial complex I, increased oxidative DNA base damage, and an increase in lipid peroxidation (reviewed by (1,5,6). The neuronal loss might be induced by necrosis or apoptosis, both processes to which oxidative stress could contribute.

Apoptosis is a morphologically distinct form of programmed cell death which involves nuclear condensation, membrane blebbing and DNA fragmentation (7). Markers of apoptotic cell death have been reported to be present in PD (8,9), although some reports are contradictory (10). Apoptosis can be induced by a variety of agents including inhibitors of mitochondrial electron transport, dopamine, β-amyloid protein, excitotoxins and ROS (11-16).

The Bcl-2 family of proteins regulates apoptosis. Bcl-2 and Bcl-x_L protect against apoptotic cell death, whereas BAX and Bcl-x_S are promoters of the death process. Bcl-2 is a 26kDa integral-membrane protein situated in the outer mitochondrial membrane, nuclear membrane and endoplasmic reticulum (17) and has often been suggested to be involved in antioxidant pathways (18). Bcl-2 is a multifunctional protein (19) with ion channel activity (20) and perhaps also a docking/binding function for positive regulators of apoptosis. When a cell decides to die a decrease in the mitochondrial membrane potential occurs (21). This leads to mitochondrial permeability transition (megapore opening) and the release of apoptotic activators, Ca^{2+} and ROS. Bcl-2 may regulate this permeability transition by enhancing the entry of ions or proteins that promote closure of the megapore, so preventing the release of these promoters of death (22). Although Bcl-2 has no

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TABLE 1

Characteristics of Patients with Their Respective Controls

Brain area	PD Age (years)	PD Post-mortem delay (hours)	Control Age (years)	Control Post-mortem delay (hours)	P values	
					Age	PM
Caudate nucleus	81.0 ± 3.0	24.0 ± 5.4	68.0 ± 5.6	33.0 ± 6.2	0.1102	0.3711
Globus pallidus	79.6 ± 2.3	21.0 ± 4.3	68.7 ± 5.6	27.0 ± 2.5	0.2235	0.1688
Putamen	78.2 ± 2.6	21.5 ± 4.1	75.2 ± 3.8	34.5 ± 6.4	0.7913	0.1212
Cortex	80.1 ± 3.0	25.0 ± 4.9	77.4 ± 2.4	34.6 ± 6.2	0.4433	0.3379
Substantia nigra	77.5 ± 3.7	27.5 ± 5.5	64.3 ± 5.8	34.5 ± 5.7	0.1747	0.6682
	ILBD	ILBD	Control	Control		
Caudate nucleus	63.0 ± 9.8	21.0 ± 1.9	61.0 ± 4.3	16.0 ± 1.3	1.000	0.1573
Globus pallidus	68.0 ± 5.8	21.0 ± 2.2	72.0 ± 4.9	14.0 ± 1.9	0.8551	0.0552
Putamen	63.0 ± 9.8	21.0 ± 1.9	69.0 ± 4.2	14.0 ± 2.4	0.7237	0.1573
Cortex	64.0 ± 5.4	19.0 ± 1.3	68.0 ± 5.7	14.0 ± 1.5	0.7842	0.0552

Note. Values are expressed as mean ± SEM. Mann-Whitney statistical test compared disease and control groups for each brain area examined.

direct antioxidant activity (Marshall, Bredesen and Halliwell, unpublished) cells over-expressing it are often protected against oxidative stress (16,23) and oxidative stress is implicated in the mitochondrial permeability transition (21).

The fact that apoptosis has been detected in neurodegenerative diseases including (8) led us to examine the levels of Bcl-2 protein in post-mortem brain samples from PD patients, especially as current literature data are conflicting, with evidence of altered expression of Bcl-2 in PD in some studies (24), but not in others (25). In addition, we examined Bcl-2 levels in brain regions from individuals with incidental Lewy body disease.

METHODS AND METHODS

Tissue preparation. This study was performed on post-mortem brain tissue from control subjects, patients dying with PD and individuals with incidental Lewy body disease. The tissue was supplied by the Parkinson's Disease Society Brain Bank and the Institute of Psychiatry. The tissue was taken from the: globus pallidus, caudate nucleus, putamen, substantia nigra, and the frontal cortex as a control area. For PD, 10 cases were supplied with 10 age and post-mortem interval matched controls. For the ILBD, 6 cases were supplied with 6 age and post-mortem interval matched controls. ILBD is defined pathologically as nigral cell loss and the presence of Lewy bodies (26,27). The ILBD cases had no known neurological illness or related treatment and came into the brain bank as controls; it was only after pathological examination that they were diagnosed as ILBD. Due to limited tissue not all brain areas were available for each patient, therefore n numbers are not always the same for each brain area examined. All PD cases were clinically and pathologically diagnosed. The controls had no known history of neurological illness and no pathological abnormalities. All brain tissue was matched for age and post-mortem delays. The brain areas were stored at −70°C until analysis.

Western blotting. Approximately 50mg of tissue was homogenised in a 100mM Tris buffer pH 6.8 containing 5% SDS, 10mM EDTA, and protease inhibitors (aprotonin 5μg/ml, leupeptin 5μg/ml, pepstatin A 7μg/ml). The homogenate was sonicated briefly (10s) and centrifuged at 4-10°C and 22,000g. The samples were kept on ice throughout the procedures. The supernatants were measured for protein content using the Lowry protein assay. Samples were diluted 1:2 with sample application buffer containing 1.5M Tris buffer, pH 6.8, 10% SDS, 0.4ml mecaptoethanol, 0.8ml glycerol and 0.2ml bromophenol blue (total 2ml). The samples were then heated at 80°C for 20mins to denature the proteins. 100μg total protein from each sample was then loaded onto a 12% polyacrylamide gel for electrophoresis. The samples were run on the gels for 1.5hr, 120volts with a standard ladder (Biorad, Hemel Hempstead, Hertfordshire) and Bcl-2 peptide control (Santa Cruz, Insight Biotechnology, Wembley). The proteins were transferred to nitro-cellulose membranes overnight at 30volts. The protein transfer was complete as determined by commassie blue staining of a duplicate gel. Membranes were washed twice in PBS and incubated for 1hr 2% FBS in PBS. After two washes with 0.1% tween in PBS the membranes were incubated for 2hrs with a mouse monoclonal anti-human bcl-2 antibody 1:100 dilution (Santa Cruz). The membranes were again washed twice with 0.1% tween in PBS and a further 1hr incubation with a goat anti-mouse antibody conjugated to alkaline phophatase (Sigma, Poole, Dorset) took place. Finally, the membranes were rinsed in PBS and washed twice in 0.05% tween in PBS. Colour development occurred through a NBT/BCIP (Sigma) substrate reaction for no longer than 5mins. The membranes were rinsed several times in water to stop the colour reaction. No non-specific binding was detected using this system. Total protein transfer onto the nitro-cellulose membranes was determined by ponceau red staining. This gave an indication of equal protein loading in each lane.

Quantification of Bcl-2 protein levels. The membranes were scanned onto disc using a Hewlett Packard Scanjet 4C (Bracknell, Berkshire). The blots were then reloaded onto a image analysis system. A direct gel analysis program was used to determine the optical densities of each band. The background absorbance of each corresponding lane was subtracted from the band levels. Disease and matched control samples were run on the same gels to allow direct comparison between groups of tissue using the non parametric Mann-Whitney statistical test. Mean age and post-mortem interval were analysed between control and disease groups for each area investigated using the same statistical test.

RESULTS

There was no significant difference in age or post-mortem interval for individual brain areas between PD

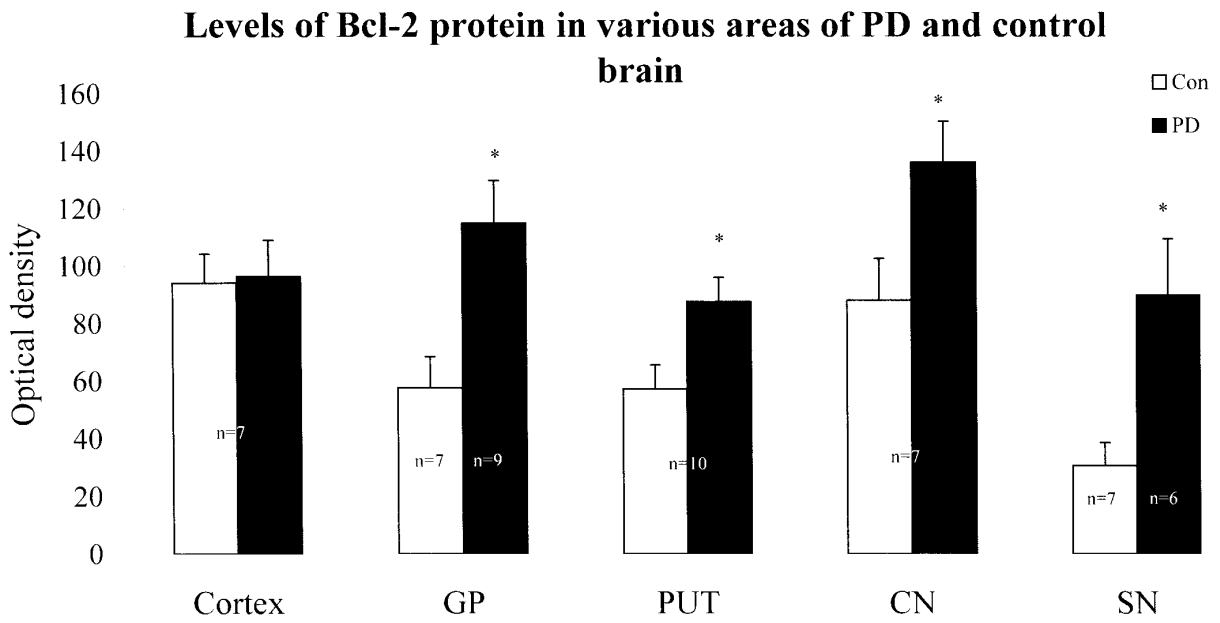

FIG. 1. Increases in Bcl-2 protein levels in the basal ganglia of parkinsonian patients as compared to age-matched controls. Optical density is expressed as arbitrary units. Values are expressed as mean ± SEM. Mann-Whitney statistical test compared disease and control groups for each brain area examined. *$p<0.05$.

or ILBD brain tissues and their matched controls (Table 1).

All areas of brain examined showed easily detectable levels of Bcl-2 protein. In PD increased Bcl-2 protein was detected as compared with control individuals in several brain areas (Fig. 1). Bcl-2 levels were significantly raised in all areas of basal ganglia, namely: substantia nigra ($p=0.018$), caudate nucleus ($p=0.041$), globus pallidus ($p=0.015$) and putamen (0.028). By contrast, Bcl-2 protein levels appeared unaltered in the frontal cortex ($p=1.00$), a region showing no pathology in PD.

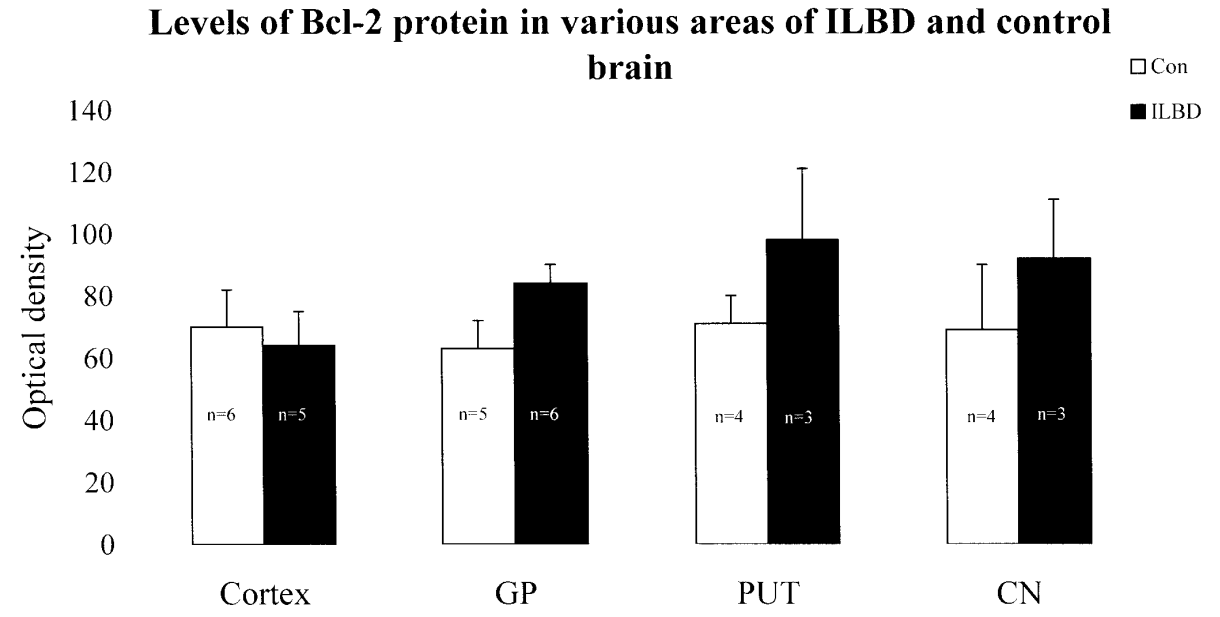

FIG. 2. Increases in Bcl-2 protein levels in the basal ganglia of incidental Lewy body disease individuals as compared to age-matched controls. Optical density is expressed as arbitrary units. Values are expressed as mean ± SEM. Mann-Whitney statistical test compared disease and control groups for each brain area examined.

In ILBD, there was also a clear trend for increased levels of Bcl-2 protein in the basal ganglia (Fig. 2). Although the increase did not reach statistical significance (caudate nucleus ($p=0.216$), putamen (0.216) and globus pallidus ($p=0.055$)), the fact that it is seen in all areas is suggestive of a real difference. Consistent with this, frontal cortex showed identical levels of Bcl-2 in ILBD and control ($p=1.00$). Regretfully, we were unable to obtain samples of substantia nigra from ILBD cases.

DISCUSSION

Our data show using western blotting that Bcl-2 protein levels are increased in PD, consistent with some previous reports (24). The increase affects areas with pathology in PD, but is not a general response of the brain because it is not seen in cerebral cortex. Our data show a trend towards an increase in Bcl-2 in ILBD, believed by many neurologists (discussed by (26)) to be presymptomatic PD.

It appears that oxidative stress may play a role in the pathology of PD, since increased lipid peroxidation, iron accumulation, superoxide dismutase activity and DNA damage (5,6) have been detected (reviewed by (1,2)). Since ROS induce apoptosis and ROS can also be involved in apoptosis induced by other mechanisms (28,29) it seems reasonable that Bcl-2 levels are increased in the substantia nigra and other basal ganglia areas. This rise in Bcl-2 supports previous work suggesting that apoptosis is involved in PD (8) and is suggestive of an attempt at an adaptive response to stress. Several parameters related to oxidative damage (e.g. protein carbonyls, rise in iron and mitochondrial defects) are not detected in ILBD cases, and thus may represent late stages in the disease and/or effects of L-DOPA therapy. By contrast, falls in GSH are seen in this condition (30), and are also associated with apoptosis (29) Although our data did not reach statistical significance, probably due to the limited number of samples available to us, the trend to a rise in Bcl-2 is clear, suggesting that it is an early event in PD and not related to L-DOPA therapy. This further implies that apoptosis may play an important role in early PD.

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