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Cell Death Mechanisms in Multiple System Atrophy

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Abstract. The presence and distribution of apoptotic cell death in multiple system atrophy (MSA) and morphologically related diseases were investigated by means of a modified terminal deoxynucleotidyl transferase-mediated nick end labeling method, comparing their distribution with that of glial cytoplasmic inclusions, immunohistochemically demonstrated bcl-2 protein, bax protein, CD95, TNFα, and p53-protein expression, as well as activated microglia. Apoptosis occurred almost exclusively in oligodendrocytes in multiple system atrophy and its general distribution was comparable to the already known oligodendroglial pathology in this disorder. Additionally, in about a quarter of glial cytoplasmic inclusions, there was upregulation of bcl-2-protein and coexpression with ubiquitin, suggesting a final attempt of involved cells to counteract apoptotic cell death. Bax protein was also demonstrated in oligodendroglial cells. A significant neuronal apoptosis was not observed in MSA; these cells might be destroyed secondarily to oligodendroglial apoptosis by necrosis or other forms of programmed cell death. These results emphasize the central role of oligodendroglial pathology in multiple system atrophy, making this disease unique among neurodegenerative diseases.

Key Words: Apoptosis; Cell death; Multiple system atrophy; Neurodegenerative diseases; Pathogenesis; Programmed cell death; TUNEL.

INTRODUCTION

By definition, the etiology of the so-called neurodegenerative diseases is still obscure. Even the exact pathogenetic steps which finally lead to neuronal cell death of certain anatomic and physiological systems in these conditions are far from clear. One of these enigmatic progressive disorders represents multiple system atrophy (MSA), a sporadic disease with onset above the age of 30 years, clinically manifested by various combinations of cerebellar, extrapyramidal, pyramidal, and autonomic symptoms (1–4). These clinical manifestations are due to neuropathological changes including variable neuronal loss, gliosis and demyelinization within the corpus striatum, substantia nigra, locus ceruleus, pontine nuclei, transverse pontine fibers, middle and inferior cerebellar peduncles, cerebellar hemispheres, Purkinje cell layer, inferior olivary nucleus, dorsal motor nucleus of vagus, nucleus vestibularis, intermediolateral cell column of spinal cord, and Onuf's nucleus (5–8). Morphologically, 2 main types may be differentiated: MSA of the olivopontocerebellar (OPCA) and striatonigral degeneration (SND) type. A further important feature of MSA in terms of diagnosis, pathogenesis and nosology, consists of predominantly oligodendroglial, argyrophilic intracytoplasmic inclusions (GCI), which are immunohistochemically expressing ubiquitin and tau protein (9–13). Therefore, the presence of both OPCA and SND together with GCIs and an appropiate clinical history, defines MSA and distinguishes it from other neurodegenerative diseases (1). Although the pathogenesis of MSA is apparently related to GCI, the underlying pathogenetic mechanisms are as yet ill-defined.

Apoptosis, a morphologically and biochemically well-characterized form of programmed cell death (PCD) (14–16), has recently been suggested as a pathogenetic event in several neurodegenerative diseases such as Parkinson (PD), Alzheimer (AD), and Huntington (HD), as well as amyotrophic lateral sclerosis (ALS) (17–23). In order to examine a possible contribution of apoptosis to the pathological picture of MSA, we investigated the presence of cells undergoing apoptosis by means of a modified terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) method comparing their distribution with that of GCI, immunohistochemically demonstrated expression of apoptosis-associated proteins such as bcl-2, bax, CD 95 (Fas/APO-1) and p53, tumor necrosis factor α (TNFα), and activated microglia in 12 cases of different multisystem degenerations including 6 cases of MSA.

MATERIALS AND METHODS

Supraspinal tissue samples of 12 patients which succumbed to progressive neurodegenerative diseases compatible with the neuropathologically confirmed clinical diagnoses of MSA (cases 1–6), infantile OPCA (cases 7–10), and autosomal dominant cerebellar ataxia type I (ADCA I) (cases 11, 12) were morphologically investigated in this study. The clinicopathological data of the cases are detailed in Table 1. In the infantile cases, a carbohydrate deficient glycoprotein deficiency syndrome (CDG) might have been the underlying disease, although its biochemical proof is lacking. Ten age-matched cases, the age of which ranged from 1 to 70 years (mean age 61 years), without neurological disease served as controls. In no case did postmortem autolysis time exceed 28 hours (h) (mean duration 15.5 h) in both the experimental and control group.

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TABLE 1
Clinical Data and Neuropathological Alterations

No.	Age	DD	Gender	FH	Symptomatology	GCI	Neuropathology
1	61	5	f	−	Cerebellar syndrome, Tetraplegia	+	OPCA
2	62	5	m	−	Cerebellar syndrome, Dysarthria, Incontinence	+	OPCA
3	65	9	m	−	Cerebellar syndrome, Incontinence, Dysarthria, Orthostatic hypotension	+	OPCA
4	66	6	f	−	Cerebellar syndrome, Parkinsonism	+	OPCA/SND
5	62	5	f	−	Parkinsonism, Spasticity, Incontinence, Dysarthria	+	SND/OPCA
6	56	6	f	−	Parkinsonism, Incontinence, Dysphagia	+	SND
7	5	4	m	+	Epilepsy, Psychomotor retardation	−	OPCA
8	4	3	f	+	Epilepsy, Psychomotor retardation, Spasticity	−	OPCA
9	1	1	f	−	Psychomotor retardation, Hepatopathy	−	OPCA
10	0.2	0.2	m	+	Epilepsy, Hepatopathy	−	OPCA
11	63	20	f	+	Cerebellar syndrome, Paraparesis, Incontinence, Ophthalmoplegia	−	AGH/OPCA
12	37	19	m	+	Cerebellar syndrome, Spasticity, Ophthalmoplegia, Dysarthria	−	AGH/OPCA

Key: m = male, f = female, DD = duration of disease in years, FH = family history, GCI = glial cytoplasmic inclusions, + = present, − = absent, OPCA = olivopontocerebellar atrophy, SND = striatonigral degeneration, AGH = neuronal loss in the spinal anterior gray horn.

TABLE 2
General Neuropathological Findings in the MSA Cases (Mean Distribution, Individual Cases May Differ)

Anatomical site	NL	Gl	Dem	GCI	TUNEL	bcl-2	bax	MG
Motor cortex	0	+		+	0	+*	(+)/+*	++
Subjacent white matter		+	+	+	0	+*	+*	++
Corpus striatum	++	++	0	+++	++*	+*	(+)/+*	++
Globus pallidus	+	+		+	0	0	+*	+
Internal capsule		++	++	+++	+++*	++*	++*	+++
Substantia nigra	++	+		++	++*	++*	(+)	++
Pontine nuclei	+++	+++		+++	(+)/++*	+++*	(+)	+++
Transverse fibers		+++	+++	+++	+++*	+++*	++*	+++
Locus ceruleus	+	+		0	+*	(+)/++*	(+)	+
Middle cerebellar peduncles		++	+++	+++	++*	++*	++*	++
Cerebellar white matter		++	+++	+++	++*	+*	++*	+++
Purkinje cell layer	++	+		0	0	0	(++)	+
Inferior olivary nucleus	+++	++	++	++	(+)/++*	+*	(+)	++
Vagal nucleus	+	+		+	0	(+)	(+)	+
Hypoglossal nucleus	+	+		+	0	(+)	(+)	+

Key: NL = nerve cell loss, Gl = astrogliosis, Dem = demyelinization, GCI = glial cytoplasmic inclusions, TUNEL = apoptotic cells, bcl-2 = cytoplasmic bcl-2-protein expression, bax = cytoplasmic bax-protein expression, MG = microgliosis consisting of HLA-DR-positive activated microglia, 0 = absent, + = few, ++ = moderate, +++ = abundant, () = neuronal, * = oligodendroglial.

Histopathology

Routinely formalin-fixed paraffin sections of all cases, covering the anatomical regions enlisted in Table 2, were stained with hematoxylin-eosin (H&E), cresyl violet, cresyl violet-Luxol fast blue, and Gallyas silver stain. Pathological changes, including neuronal loss, gliosis, and demyelinization were assessed independently by 3 of the authors and the mean of their semi-quantitative assessment was defined as follows: 0 = normal, + = mild, ++ = moderate, +++ = severe.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm-thick sections according to the avidin-biotin-peroxidase complex (ABC)- and alkaline phosphatase-anti alkaline phosphatase (APAAP)- methods using diaminobenzidine (DAB) and Fast Red (FR), respectively, as a chromogen. Primary antibodies against ubiquitin (Anti-Ubiquitin, polyclonal, 1:800, DAKO), tau protein (Anti-Tau, polyclonal, 1:200, DAKO), glial fibrillary acidic protein (Anti-GFAP, polyclonal, 1:400, DAKO), calbindin (Anti-Calbindin-D, monoclonal, 1:1,000, Sigma), human leucocyte antigen-DR (Anti-HLA-DR, CR3/43, monoclonal, 1:100, DAKO), Leu7/CD57 (Anti-CD57, monoclonal, undiluted, Becton Dickinson), leucocyte common antigen (Anti-LCA, monoclonal, 1:150, DAKO), CD68 (Anti-CD68, KP1, monoclonal, 1:100, DAKO), neurofilament protein (Anti-NF, 2F11, monoclonal, 1:200, DAKO), bcl-2 protein (Anti-bcl-2, monoclonal, 1:100, DAKO), CD95 (Fas/APO-1) (Anti-CD95, monoclonal, 1:10, DAKO), TNFα (Anti-TNFα, polyclonal, 1:10,000, Genzyme), p53 protein (Anti-p53, polyclonal, 1:50, DAKO), and bax protein (Anti-bax, polyclonal, 1:10, BioGenex) were applied. The regional expression of these antigens was also graded by consensus of the examiners as follows: 0 = absent, + = few, ++ = moderate, +++ = abundant. In the GCI-positive MSA cases, double labeling of GCI with the antibodies to ubiquitin and bcl-2 was carried out as well. Additionally, mirror-sections were performed to compare ubiquitin- and bax-expression. Negative control sections omitting the primary antibody failed to develop specific staining.

In-situ-detection of DNA Fragmentation

For the detection of cells undergoing apoptotic cell death, in-situ-labeling was performed using a commercially available kit (ApopTagPlus Kit, ONCOR, Gaithersburg, Md., USA) according to product specifications. This recently established (24), modified TUNEL-assay has been described in detail elsewhere (25–31). In principle, by Ca^{2+}/Mg^{2+}-dependent endonuclease DNA degradation newly generated 3′-OH ends of nucleosome sized DNA fragments are catalytically tailed by TdT with digoxigenin-conjugated nucleotides, which in turn are recognized by an anti-digoxigenin antibody with peroxidase or alkaline phosphatase conjugate. In a final step, a color reaction is achieved by ABC or APAAP methods with either DAB or Fast Red as chromogen. Again, the number of apoptotic cells was graded by consensus as follows: 0 = absent, + = few, ++ = moderate, +++ = abundant. Negative controls were performed by substituting distilled water for TdT enzyme in the preparation of the working solution. Since DNA fragmentation also occurs in necrosis, only single cells with significant nuclear labeling were considered as apoptotic. In this setting, experience gained with the TUNEL method over the last years suggests that it represents a reliable approach for the detection of apoptosis, especially when combined with other methods, such as DNA laddering as seen by electrophoresis or immunohistochemistry for apoptosis-related antigens (24). We also investigated the expression of apoptosis-related proteins, such as bcl-2, bax, CD95, and p53 to corroborate the TUNEL results. In order to further characterize TUNEL-positive cells, TUNEL was combined with immunohistochemistry for Leu7/CD57, LCA, GFAP, CD68, and NF.

RESULTS

Although severity and distribution of histopathological changes, immunohistochemical reactions and TUNEL-staining varied from brain to brain, their distribution pattern within the groups appeared uniform, justifying the comprehensive presentation of the morphologic observations of the MSA cases in Table 2. Results of the non-MSA cases will be briefly presented in the text.

Histopathology

The distribution of nerve cell loss, gliosis, demyelinization, and the presence of GCI allowed the differentiation of the cases into the following groups: MSA-OPCA (cases 1–4), MSA-SND (cases 5, 6), infantile OPCA (cases 7–10), and ADCA I (cases 11, 12). The infantile cases presented a pure OPCA syndrome with nerve cell loss in the inferior olivary nuclei, pontine nuclei and Purkinje cells only, which would be compatible with a CDG-syndrome. GCI were not observed. The adult hereditary cases showed more widespread pathological changes, including nerve cell loss and gliosis in the substantia nigra, oculomotor nucleus, nuclei pontis, cerebellar cortex, inferior olivary nucleus, vagal and hypoglossal nuclei, and spinal anterior gray horn, thus most likely representing cases of ADCA I. GCI were absent as well. In contrast, Gallyas staining disclosed abundant GCI in the MSA cases, exclusively.

Immunohistochemistry

Ubiquitin-positive, tau-expressing GCI were present in all cases of MSA, whereas they were consistently absent from ADCA I- and infantile OPCA-cases as well as from normal controls. GCI-distribution did not strongly reflect nerve cell loss and demyelinization, thus keeping in line with previous reports (11, 13). Areas with the highest density include the white matter underlying the motor cortex, corpus striatum, internal capsule, substantia nigra, basis pontis, cerebellar hemispheres, inferior olivary nucleus, and the dorsal motor nucleus of vagus. GFAP-positivity disclosed gliosis in regions of pathological changes in all cases. Calbindin was selectively expressed both in Purkinje-cells and in neurons of the inferior olivary nucleus, thus facilitating the demonstration of nerve cell loss in these populations (Fig. 1). Pronounced in the white matter, abundant HLA-DR-positive activated microglial cells were present in all but 2 infantile OPCA cases, disclosing and indicating more widespread lesions than observed in conventional stains. The distribution of HLA-DR-positive activated microglia reflected the distribution of both demyelinization and neuronal death (Fig. 2). This microgliosis seemed to be a rather unspecific reaction to injury, independent from the mode of cell death. All cases, including normal controls, showed a strong bcl-2 expression in the ependymal cell layer, a moderate expression in vascular lymphocytes, and finally a weak, age-dependently decreasing neuronal cytoplasmic positivity, particularly in the locus ceruleus. In the MSA cases, however, a distinct cytoplasmic demonstration of bcl-2 was observed in oligodendroglial cells in a GCI-like shape. Double labeling revealed coexpression of ubiquitin and bcl-2 in about a quarter of the GCI (Fig. 3). Inconclusive weak staining was observed with the antibody to TNFα only in glial cells and some endothelial

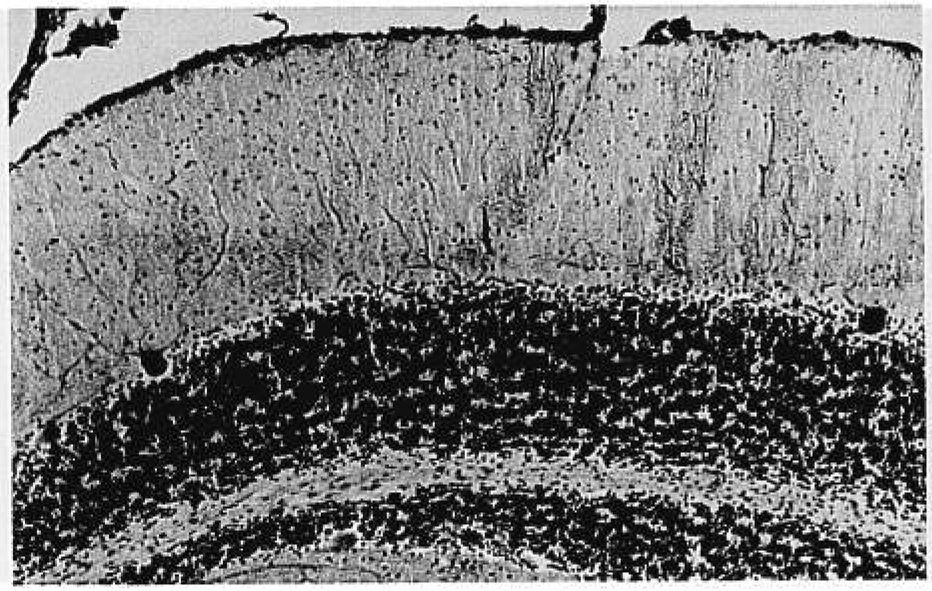

Fig. 1. MSA case. Calbindin immunostaining demonstrates Purkinje cell loss between 2 remaining neurons (Anti-Calbindin, ABC, DAB, ×32).

cells without any linkage to pathological alterations. No expression of either CD95 or p53-protein was seen in any investigated case. Bax protein was detected in different neuronal populations, particularly in Purkinje cells and both cortical and brainstem motor neurons. This expression did not significantly differ between MSA cases and controls. Characteristic for the MSA cases, however, was a moderate cytoplasmic oligodendroglial bax-expression (Fig. 4), especially in areas in which also oligodendroglial DNA-fragmentation and bcl-2 expression occurs. Because of polyclonality of both antibodies, mirror sections did not suggest a colocalization of bax and ubiquitin-positive GCI.

TUNEL-labeling

A significant DNA fragmentation characteristic of apoptosis was detected in all but 1 infantile OPCA case and the normal controls. The vast majority of cells undergoing apoptosis in the MSA cases, as demonstrated by TUNEL, represented interfascicular (Fig. 5A) and perineuronal (Fig. 5B) oligodendrocytes. The identification of these cells as oligodendrocytes was further strengthened (besides their typical morphological shape and topographical distribution) by their expression of Leu7/CD57 (9) (Fig. 5C). Additionally, coexpression of LCA, GFAP, NF or CD68 in these TUNEL-positive cells was absent. The distribution of these apoptotic cells was variable and did not reflect nerve cell loss in individual cases. The correlation of apoptotic cells to both the presence of GCI and activated microglia in a given case was also poor, as illustrated in this case (Fig. 2), where a dramatic microgliosis lacked a correlation with GCI or apoptotic cells. In general, however, it seemed that areas known to harbor many GCI were rich in apoptotic oligodendrocytes. Pontine nuclei were moderately affected in 1 MSA-OPCA, and 1 ADCA I case, whereas they were significantly involved in merely 2 infantile OPCA cases. Inferior olivary nuclear neurons showed a weak nuclear positivity in 2 cases (1 MSA-SND, 1 ADCA I) only. Interestingly, Purkinje cells as well as other neurons consistently lacked features of apoptosis by TUNEL in all cases.

DISCUSSION

As we have previously reported (32), apoptotic cell death occurs in MSA as well as in morphologically related but nosologically and etiologically different disorders such as ADCA I and infantile OPCA. The death of specific neuronal subpopulations due to ill-defined mechanisms on the basis of equally unclear etiologies is seen in MSA, ADCA-1 and OPCA as well as in other neurodegenerative diseases such as AD, ALS, HD, and PD. Several hypotheses have been proposed to explain the mechanisms that might underlie these diseases, e.g. the lack of or defects in trophic interactions between the neurons and their targets (33), the excess of glutamate and calcium (34), oxygen free radicals (35), autoimmunity (36) or combinations of the above (37). Whatever the underlying mechanisms, there is increasing evidence that 1 possible final pathway of cell death occurring in these diseases is PCD (17). Thus, tissue specimens taken from patients with AD, ALS, HD, and PD revealed biochemical processes and nick end labeling features suggestive of apoptosis (19, 20, 21, 38, 39). Whereas most of the studies demonstrated apoptosis of the involved neuronal populations, some also noted significant apoptotic cell

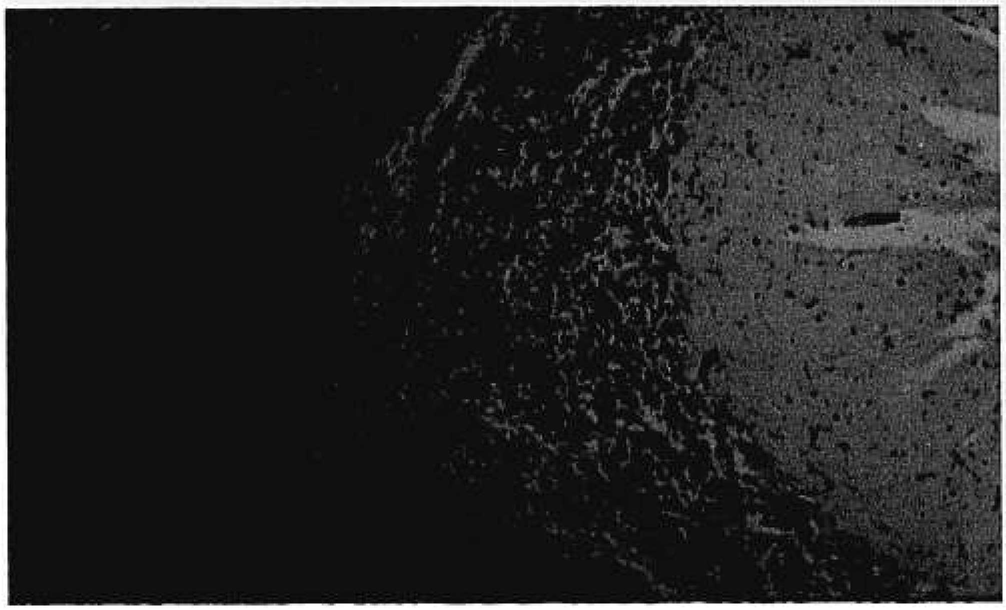

Fig. 2. MSA case. Cerebellum: Abundant HLA-DR-expressing activated microglial cells in the white matter. In the same area only a very few GCI and no apoptotic cells were seen (not shown) (CR 3/43, ABC, DAB, ×32).

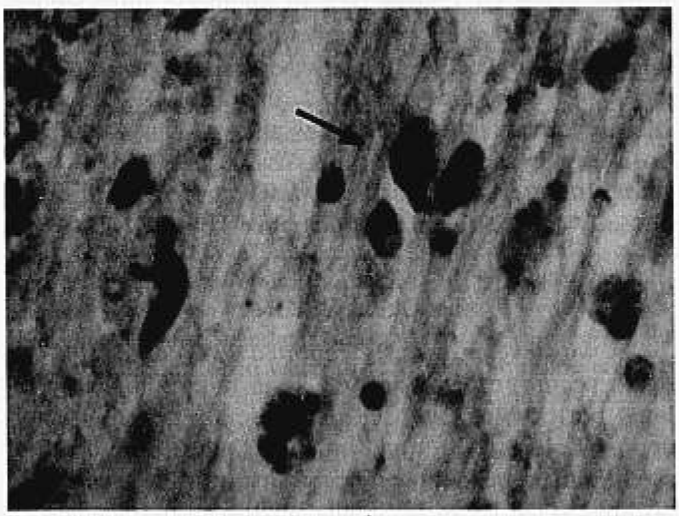

Fig. 3. MSA case. Double labeling of a pontine GCI coexpressing ubiquitin (brown) and bcl-2 (red) (arrow). Compare with ubiquitin-positive axon and GCI in the vicinity (brown only) (Anti-Ubiquitin, ABC, DAB, Anti-bcl-2, APAAP, FR, ×201).

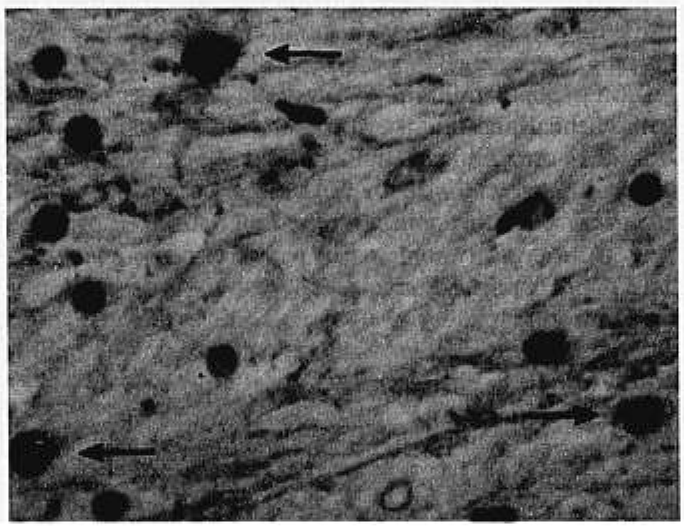

Fig. 4. MSA case. Cytoplasmic bax-expression is shown in some oligodendrocytes (arrows) (Anti-bax, ABC, DAB, ×201).

death in oligodendroglial and microglial cells (19, 20, 40). Apoptosis of oligodendroglial cells has been previously demonstrated in up to 50% of oligodendrocytes during normal development (41), in multiple sclerosis (42), following spinal cord trauma (43), and in models of HTLV-1-encephalitis (44). Thus, among glial cells, oligodendrocytes appear to be particularly vulnerable to PCD via apoptosis (23). However, unlike the above mentioned neurodegenerative diseases, in MSA apoptotic cell death affects almost exclusively oligodendroglial cells in a distribution pattern similar to that of GCI, which are currently regarded as the predominant and primary cellular lesion (12, 45).

Further evidence in favor of this notion may be our observation of bcl-2 expression in GCI. This apoptosis-related protein is an integral mitochondrial membrane protein playing a central role in the inhibition of apoptosis, presumably by interfering with reactive oxygen molecules (46). Bcl-2 is widely expressed in the developing nervous system, suggesting that many immature and later post–mitotic cells require a death repressor molecule (47–49). Postnatally, its neuronal expression de-

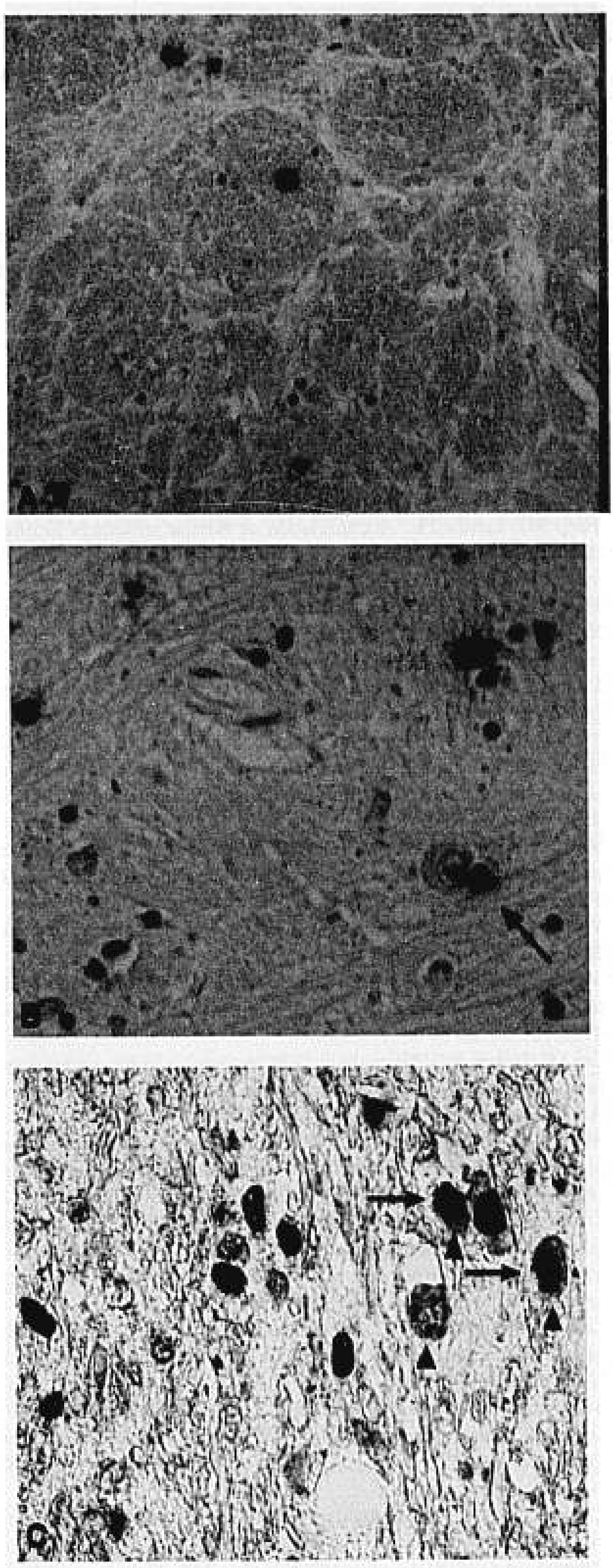

Fig. 5. MSA case. A) Basal ganglia: TUNEL positive apoptotic interfascicular oligodendrocytes in fibers of the internal capsule (ApopTagPlus, APAAP, FR, ×64). B) Basal ganglia: Perineuronal satellite oligodendrocyte undergoing apoptotic cell death (arrow) (ApopTagPlus, APAAP, FR, ×128). C) Cerebellar ← white matter: TUNEL positive nuclei (arrows) in cells with cytoplasmic expression of Leu7/CD57 (arrowheads) (ApopTagPlus, APAAP, FB, Anti-Leu7/CD57, ABC, FR, ×201).

clines with age to hardly detectable levels which contribute to promote neuronal survival throughout adult life. In normal brain only ependymal cells retain a strong bcl-2 expression (49, 50). Oligodendrocytes, however, both in normal and pathological conditions are generally reported to remain bcl-2 negative, which partially explains their vulnerability to apoptosis (23, 43, 51, 52). The demonstration of bcl-2 in pathologically altered oligodendrocytes in MSA, as particularly indicated by bcl-2 positive GCI, might thus represent a final repair mechanism of a sublethally damaged cell to avoid induced PCD via apoptosis by the upregulation of this anti-apoptotic protein.

The mechanisms responsible for the initiation of oligodendroglial apoptosis in MSA are not known. So far, according to our results, we have no evidence that this apoptosis is triggered via the p53-protein- or the CD95-pathway, nor that TNFα might significantly contribute to it. However, there is oligodendroglial expression of the pro-apoptotic protein bax, which has previously only been described in neuronal elements, microglia and ependymal cells in normal and pathological brains (52–54). Unlike bcl-2 expression, we were unable to demonstrate a coexpression of bax with GCI on mirror sections. Thus, expression or upregulation of bax might not be directly influenced by or related to GCI-formation and possibly represents a different stage of oligodendroglial pathology. However, bax-positivity in oligoglial cells further corroborates the finding of oligodendroglial apoptosis as an important cell death mechanism in MSA, as indicated by TUNEL, and also underscores the central role of oligodendroglial pathology in this disorder. The apoptotic oligodendrocytes—in analogy to GCI, which might represent a prestage—may thus be interpreted as an early phenomenon preceding neuronal degeneration. However, at present, the possibility can not be entirely ruled out that they reflect the consequence of neuronal death and withdrawal of axon-derived trophic factors normally preventing oligodendroglial apoptosis. Further studies are urgently needed to clarify this issue.

Since apoptosis does not seem to significantly contribute to neuronal cell death in MSA, it is possible that the majority of dying nerve cells are destroyed either by necrosis or by a different form of PCD other than apoptosis. In this regard, MSA represents a unique form of neurodegenerative disease in which hitherto unknown etiological (environmental, genetic) factors presumably induce a tau-protein- or α- and β-tubulin- related cytoskeletal pathology of oligodendrocytes (45). These cells upregulate bcl-2 protein in order to counteract apoptosis but finally succumb to the disease process when upregulation of bax protein outweights these efforts leading to oligoglial apoptosis, which eventually proceeds via the myelin-axon complex to neuronal degeneration of the suprasegmental motor and supraspinal autonomic system. A common final pathway may then represent the sometimes extensive activation of microglial cells which contributes to both demyelinization and neuronal removal, independently from the underlying mode of cell death.

Apoptotic cell death is now emerging as an important and widespread phenomenon of cell death in disease, and is not only limited to its well-established role during development. The extent to which apoptosis is involved in various neurological disorders such as MSA and the resulting implications, however, are still not clear. Since apoptosis is morphologically different from necrosis, this implies an underlying mechanistic difference between the 2 processes, and therefore blocking apoptosis might someday provide another potential course for therapeutic intervention (17). Based on the experiences gained by prevention of apoptosis in sympathetic and motor neurons by either manipulation of the bcl-2 family or inhibition of caspases, it may be speculated that in a situation such as a trauma or stroke, during which cell death may occur over days to a few weeks and where reversal of some of the processes (blood flow alterations, inflammation) will reestablish a normal environment, anti-apoptotic therapies may prove useful. Such interventions would probably keep neurons and glial cells alive until death-inducing stimuli subside. However, chronic neurodegenerative diseases such as MSA present a different problem. In these situations, if the ultimate demise of the cell involves apoptosis, the apoptotic event is the result of a chronic derangement that has already led to a metabolically and structurally altered cell in a hypo-, non-, or dysfunctional state. In this case, anti-apoptotic therapy alone, without influencing underlying etiological factors, may be of less utility as such therapies do not reverse the events that have led to this dysfunctional state. Preventing the apoptosis of such cells may thus prove to be of little functional consequence (55).

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